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**WO 03/011334 A1**

(54) Title: VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNISATION AGAINST HIV

(57) Abstract: Use of a) an HIV Tat protein or polynucleotide; or b) an HIV Nef protein or polynucleotide; or c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide; and an HIV gp 120 protein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV, wherein the protein or polynucleotide is delivered via a bombardment approach.

5 VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNISATION AGAINST HIV

## DESCRIPTION

The present invention relates to novel uses of HIV proteins in medicine and vaccine  
10 compositions containing such HIV proteins. In particular, the invention relates to the  
use of HIV Tat and HIV gp120 proteins in combination. Furthermore, the invention  
relates to the use of HIV Nef and HIV gp120 proteins in combination. The invention  
also relates to DNA encoding HIV Tat and/or Nef (hereinafter Tat DNA and/or Nef  
DNA) and DNA encoding HIV gp120 (hereinafter gp120 DNA) and vectors  
15 comprising such DNAs. The invention relates in particular to administering the  
proteins and/or DNAs in a prime-boost schedule via a particle bombardment  
approach.

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS)  
20 which is regarded as one of the world's major health problems. Although extensive  
research throughout the world has been conducted to produce a vaccine, such efforts  
thus far have not been successful.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment  
25 to the host cell. This attachment is mediated by the binding to two surface molecules  
of helper T cells and macrophages, known as CD4 and one of the two chemokine  
receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger  
precursor molecule (gp160), which is then cleaved post-translationally to yield gp120  
and gp41. The gp120 protein is retained on the surface of the virion by linkage to the  
30 gp41 molecule, which is inserted into the viral membrane.

The gp120 protein is the principal target of neutralizing antibodies, but unfortunately  
the most immunogenic regions of the proteins (V3 loop) are also the most variable  
parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine  
35 antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly  
protective vaccine. The gp120 protein does also contain epitopes that are recognized  
by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-

5 infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

10

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), *Pediatr. Infect. Dis. J.*, 11, 5, 15 390 et seq (1992).

HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

20 In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12<sup>th</sup> Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and 25 one dose recombinant protein). However, the results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

However, we have found that a Tat- and/or Nef-containing immunogen (especially a 30 Nef-Tat fusion protein) acts synergistically with gp120 in protecting rhesus monkeys from a pathogenic challenge with chimeric human-simian immunodeficiency virus (SHIV). To date the SHIV infection of rhesus macaques is considered to be the most relevant animal model for human AIDS. Therefore, we have used this preclinical model to evaluate the protective efficacy of vaccines containing a gp120 antigen and a 35 Nef- and Tat-containing antigen either alone or in combination. Analysis of two markers of viral infection and pathogenicity, the percentage of CD4-positive cells in the peripheral blood and the concentration of free SHIV RNA genomes in the plasma

5 of the monkeys, indicated that the two antigens acted in synergy. Immunization with either gp120 or NefTat + SIV Nef alone did not result in any difference compared to immunization with an adjuvant alone. In contrast, the administration of the combination of gp120 and NefTat + SIV Nef, antigens resulted in a marked improvement of the two above-mentioned parameters in all animals of those particular  
10 experimental group.

As described above, the NefTat protein, the SIV Nef protein and gp120 protein together give an enhanced response over that which is observed when either NefTat + SIV Nef, or gp120 are used alone. This enhanced response, or synergy can be seen in  
15 a decrease in viral load as a result of vaccination with these combined proteins. Alternatively, or additionally the enhanced response manifests itself by a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV NefTat, SIV Nef and HIV gp120. The synergistic effect is attributed to the combination of gp120 and Tat, or gp120 and Nef, or gp120 and both Nef and Tat.

20 It has been found that not only the Nef, Tat or NefTat proteins are advantageously combined or administered with the gp 120 protein. The same advantages are seen when DNA encoding Nef, Tat or NefTat is administered with gp 120 (protein or corresponding DNA).

25 It has been found that the proteins above, or DNA encoding the proteins, may advantageously be administered via a prime-boost strategy. In one aspect the present invention relates to such administration via a bombardment approach. Accordingly the invention provides the use of

- 30 a) an HIV Tat protein or polynucleotide; or  
b) an HIV Nef protein or polynucleotide; or  
c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;  
and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable  
35 for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV, wherein the protein or polynucleotide is delivered via a bombardment approach.

5

Numerous methods of carrying out a particle bombardment approach are known. See for example WO 91/07487. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI),  
10 some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, coated with a substance such as polynucleotide, is accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest,  
15 typically the skin. The particles are preferably gold beads of a 0.4 – 4.0  $\mu\text{m}$ , more preferably 0.6 – 2.0  $\mu\text{m}$  diameter and the polynucleotide, preferably DNA is coated onto these and then encased in a cartridge for placing into the “gene gun”.

The addition of other HIV proteins or DNA encoding them may further enhance the synergistic effect, which was observed between gp120 and Tat and/or Nef. These  
20 other proteins may also act synergistically with individual components of the gp120, Tat and/or Nef-containing vaccine, not requiring the presence of the full original antigen combination. The additional proteins may be regulatory proteins of HIV such as Rev, Vif, Vpu, and Vpr. They may also be structural proteins derived from the HIV  
25 *gag* or *pol* genes.

The HIV *gag* gene encodes a precursor protein p55, which can assemble spontaneously into immature virus-like particles (VLPs). The precursor is then proteolytically cleaved into the major structural proteins p24 (capsid) and p18  
30 (matrix), and into several smaller proteins. Both the precursor protein p55 and its major derivatives p24 and p18 may be considered as appropriate vaccine antigens which may further enhance the synergistic effect observed between gp120 and Tat and/or Nef. The precursor p55 and the capsid protein p24 may be used as VLPs or as monomeric proteins.

35

The HIV Tat protein for use in the present invention may, optionally, be linked to an HIV Nef protein, for example as a fusion protein.

5

The HIV Tat protein, the HIV Nef protein or the NefTat fusion protein for use in the present invention may have a C terminal Histidine tail which preferably comprises between 5-10 Histidine residues. The presence of an histidine (or 'His') tail aids purification.

10

In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (*Saccharomyces cerevisiae*), of Nef (Macreadie I.G. et al., 1993, *Yeast* 9 (6) 565-573) and Tat (Braddock M et al., 1989, *Cell* 58 (2) 269-79) has been reported. Nef protein and the Gag proteins p55 and p18 are myristoylated. The expression of Nef and Tat separately in a *Pichia* expression system (Nef-His and Tat-His constructs), and the expression of a fusion construct Nef-Tat-His have been described previously in WO99/16884.

15

20

The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-His (Seq. ID. No.s 10 and 11) and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13) are set forth in Figure 1.

25

The HIV proteins may be used in their native conformation, or more preferably, may be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the Tat or Nef protein. Thus the invention encompasses the use of derivatives of HIV proteins or polynucleotides, particularly DNAs, which may be, for example, mutated. The term 'mutated' is used herein to mean a DNA or protein molecule which has undergone deletion, addition or substitution of one or more nucleotides or amino acids using well known techniques for site directed mutagenesis or any other conventional method.

30

35

For example, a mutant Tat protein may be mutated so that it is biologically inactive whilst still maintaining its immunogenic epitopes. One possible mutated tat gene, constructed by D.Clements (Tulane University), (originating from BH10 molecular

5 clone) bears mutations in the active site region (Lys41→Ala) and in RGD motif (Arg78→Lys and Asp80→Glu) ( Virology 235: 48-64, 1997).

A mutated Tat is illustrated in Figure 1 (Seq. ID. No.s 22 and 23) as is a Nef-Tat Mutant-His (Seq. ID. No.s 24 and 25).

10

The HIV Tat or Nef proteins for use in the present invention may be modified by chemical methods during the purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a protein such as Tat or Nef is the use of chemical modifications of the protein's thiol groups. In a first step  
15 the disulphide bridges are reduced by treatment with a reducing agent such as DTT, beta-mercaptoethanol, or glutathione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (for example, the protein can be caused to react with iodoacetamide). Such chemical modification does not modify functional properties of Tat or Nef as assessed by cell binding assays and inhibition of  
20 lymphoproliferation of human peripheral blood mononuclear cells.

It will be understood that the invention also encompasses the use of fragments of the full length proteins provided that the fragments comprise at least one immunogenic epitope.

25

The HIV Tat protein and HIV gp120 proteins can be purified by the methods outlined in the attached examples.

An immunoprotective or immunotherapeutic quantity of the Tat and/or Nef or NefTat  
30 and gp120 components (protein or DNA) for use in the invention may be prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A.  
35 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for

5 example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in a vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical  
10 vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 µg of each protein, preferably 2-200 µg, most preferably 4-40 µg of Tat or Nef or NefTat and preferably 1-150 µg, most preferably 2-25 µg of gp120. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of  
15 antibody titres and other responses in subjects. One particular example of a vaccine dose will comprise 20 µg of NefTat and 5 or 20 µg of gp120. Following an initial vaccination, subjects may receive a boost in about 4 weeks, and a subsequent second booster immunisation.

20 The proteins of the present invention are preferably adjuvanted in a vaccine formulation of the invention. The polynucleotides used in the present invention are optionally adjuvanted, and may be delivered in a formulation with an adjuvant or separately from the adjuvant, either simultaneously or sequentially. Adjuvants are described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited  
25 by Powell and Newman, Plenum Press, New York, 1995.

Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or  
30 anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of vaccines for use in the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

35 An immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. The resultant immune response may be broadly



5 distinguished into two extreme categories, being humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

10

Extreme Th1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice Th1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type  
15 antibodies. Th2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

20

It can be considered that the driving force behind the development of these two types of immune responses are cytokines, a number of identified protein messengers which serve to help the cells of the immune system and steer the eventual immune response to either a Th1 or Th2 response. Thus high levels of Th1-type cytokines tend to  
favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

25

It is important to remember that the distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in  
30 murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, Th1-type responses are associated with the production of the INF- $\gamma$  and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the  
35 induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor- $\beta$ (TNF- $\beta$ ).

5

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2 - type cytokine responses. Traditionally the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection includes direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*, and induces antigen specific immunoglobulin responses associated with Th1-type isotype.

Preferred Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

20

Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, *Immunology*, 60(1):141-6; and EP 0 549 074 B1). A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2µm in diameter, and its method of manufacture is disclosed in EP 0 689 454.

Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. *Phytomedicine* vol 2 pp 363-386). For example, Quil A (derived from the bark of the

5 South American tree *Quillaja Saponaria Molina*), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US  
10 Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. *J. Immunology* vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems  
15 comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for  
20 cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al.*, *J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic  
25 oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, *Nature* 374, p546 1995. Detailed analysis has  
30 shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

35

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different

5 motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon  $\gamma$  and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have  
10 also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as  
15 aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al.*, *Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including  
20 aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan  
25 *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the  
30 combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of  
35 monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in

- 5 WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred  
10 formulation for use in the invention.

Another preferred formulation comprises a CpG oligonucleotide alone or together with an aluminium salt.

15 Particularly preferred adjuvant and/or carrier combinations are as follows:

- i) 3D-MPL + QS21 in DQ
- ii) Alum + 3D-MPL
- iii) Alum + QS21 in DQ + 3D-MPL
- iv) Alum + CpG
- 20 v) 3D-MPL + QS21 in DQ + oil in water emulsion
- vi) CpG

As already noted, the vaccine may contain polynucleotide, preferably DNA, encoding one or more of the Tat, Nef and gp120 polypeptides, such that the polypeptide is  
25 generated *in situ*.

The DNA constructs *per se*, especially those described herein, also form part of the invention.

30 The polynucleotide may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems such as plasmid DNA, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, Crit. Rev. Therap. Drug Carrier Systems 15:143-198, 1998 and references cited therein,  
35 incorporated herein by reference.

5 Plasmid based delivery of genes, particularly for immunisation or gene therapy purposes is known. For example, administration of naked DNA by injection into mouse muscle is outlined by Vical in International Patent Application WO90/11092.

Johnston et al WO 91/07487 describe methods of transferring a gene to vertebrate  
10 cells, by the use of microprojectiles that have been coated with a polynucleotide encoding a gene of interest, and accelerating the microparticles such that the microparticles can penetrate the target cell.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a  
15 strong viral promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequences. The gene of interest may encode a full protein, a fusion protein comprising different antigens, or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which is intended to be protected against. Thus the plasmid may encode a fragment of a full  
20 protein, provided that the fragment comprises at least one immunogenic epitope of the full protein. The plasmid can be grown in bacteria, such as for example E.coli and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the plasmid is taken up by cells of the host where the encoded peptide  
25 is produced. The plasmid vector will preferably be made without an origin of replication which is functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned. All of these features may apply singly or in combination to the present invention.

30

There are a number of advantages of DNA vaccination relative to traditional vaccination techniques. First, it is predicted that because the proteins which are encoded by the DNA sequence are synthesised in the host, the structure or conformation of the protein will be similar to the native protein associated with the  
35 disease state. It is also likely that DNA vaccination will offer protection against different strains of a virus, by generating a cytotoxic T lymphocyte response that recognises epitopes from conserved proteins. Furthermore, because the plasmids are

- 5 taken up by the host cells where antigenic protein can be produced, a long-lasting immune response will be elicited. The technology also offers the possibility of combining diverse immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.
- 10 Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

In one preferred embodiment the DNA can be delivered via a particle bombardment  
15 approach e.g. a "gene gun" approach as described hereinabove.

Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). When the expression system is a recombinant live microorganism, such as a virus or  
20 bacterium, the gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g. vaccinia, fowlpox, canarypox, modified poxviruses e.g. Modified Virus Ankara (MVA)), alphaviruses  
25 (Sindbis virus, Semliki Forest Virus, Venezuelan Equine Encephalitis Virus), flaviviruses (yellow fever virus, Dengue virus, Japanese encephalitis virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), *Listeria*, *Salmonella*, *Shigella*, *Neisseria*, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in  
30 order to obtain live vaccines.

Thus, the Nef, Tat and gp120 components of a preferred vaccine according to the invention may be provided in the form of polynucleotides or recombinant DNA encoding the desired proteins. The polynucleotides employed in the invention may  
35 encode a full protein, a fusion protein comprising different antigens, or one or more antigenic peptide sequences. Thus the polynucleotides may encode a fragment of a

- 5 full protein, provided that the fragment comprises at least one immunogenic epitope of the full protein.

At least one of the DNAs for Nef, Tat, NefTat or gp 120 may preferably be codon optimised as described, for example, in Andre S. Seed B. Eberle J. Schraut W.

- 10 Bultmann A. Haas J. (1998): Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage, Journal of Virology. 72(2):1497-503. In one preferred aspect the DNA encoding gp120 is codon optimised.

- 15 Codon optimisation is used to optimise the polynucleotide sequences for expression in mammalian cells. That is the sequence is optimised to resemble the codon usage of genes in mammalian cells.

- In one embodiment of the present invention the Nef, Tat, NefTat or gp120  
20 polynucleotide sequence has a codon usage pattern which resembles that of highly expressed mammalian genes, particularly human genes. Preferably the polynucleotide sequence is a DNA sequence. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

- 25 The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encodes in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons  
30 representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

- Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different  
35 species show a different bias in their codon selection and, furthermore, utilisation of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and



5 mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in E.coli or a viral gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient  
10 expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons which are rarely observed in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

In the polynucleotides of the present invention, the codon usage pattern is altered from  
15 that typical of human immunodeficiency viruses to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura  
20 et.al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus  
25 each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the  
30 total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

35 According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude rare codons representing less than 10% of the codon use for an

- 5 amino acid in highly expressed genes of the target organism. In an alternative preferred embodiment, the polynucleotide will exclude codons with an RSCU value of less than 0.2 in highly expressed genes of the target organism. A relative synonymous codon usage (RSCU) value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. A
- 10 polynucleotide of the present invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. Codon usage tables for human can also be found in Genebank.
- 15 In comparison, a highly expressed beta action gene has a RSCU of 0.747. The codon usage table for a *homo sapiens* is set out below:

Codon Usage Table:

20 ***Homo sapiens* [gbpri]: 27143 CDS's (12816923 codons) Standard Codon Usage Table**

fields: [triplet] [frequency: per thousand] ([number])

25	UUU 17.0 (217684)	UCU 14.8 (189419)	UAU 12.1 (155645)	UGU 10.0 (127719)
	UUC 20.5 (262753)	UCC 17.5 (224470)	UAC 15.8 (202481)	UGC 12.3 (157257)
	UUA 7.3 ( 93924)	UCA 11.9 (152074)	UAA 0.7 ( 9195)	UGA 1.3 ( 16025)
	UUG 12.5 (159611)	UCG 4.5 ( 57572)	UAG 0.5 ( 6789)	UGG 12.9 (165930)
30	CUU 12.8 (163707)	CCU 17.3 (222146)	CAU 10.5 (134186)	CGU 4.6 ( 59454)
	CUC 19.3 (247391)	CCC 20.0 (256235)	CAC 14.9 (190928)	CGC 10.8 (137865)
	CUA 7.0 ( 89078)	CCA 16.7 (214583)	CAA 12.0 (153590)	CGA 6.3 ( 80709)
	CUG 39.7 (509096)	CCG 7.0 ( 89619)	CAG 34.5 (441727)	CGG 11.6 (148666)
35	AUU 15.8 (202844)	ACU 12.9 (165392)	AAU 17.0 (218508)	AGU 12.0 (154442)
	AUC 21.6 (277066)	ACC 19.3 (247805)	AAC 19.8 (253475)	AGC 19.3 (247583)
	AUA 7.2 ( 92133)	ACA 14.9 (191518)	AAA 24.0 (308123)	AGA 11.5 (147264)
	AUG 22.3 (285776)	ACG 6.3 ( 80369)	AAG 32.6 (418141)	AGG 11.3 (145276)
40	GUU 10.9 (139611)	GCU 18.5 (236639)	GAU 22.4 (286742)	GGU 10.8 (138606)
	GUC 14.6 (187333)	GCC 28.3 (362086)	GAC 26.1 (334158)	GGC 22.7 (290904)
	GUA 7.0 ( 89644)	GCA 15.9 (203310)	GAA 29.1 (373151)	GGA 16.4 (210643)
	GUG 28.8 (369006)	GCG 7.5 ( 96455)	GAG 40.2 (515485)	GGG 16.4 (209907)

- 5 Coding GC 52.51% 1st letter GC 56.04% 2nd letter GC 42.35% 3rd letter GC 59.13%.

According to a further aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a Nef and/or Tat or NefTat,  
10 and a gp120 polynucleotide sequence according to the first aspect of the invention, in particular where the codon usage pattern of at least one of the Nef, Tat, NefTat or gp120 polynucleotide sequences, particularly the gp120 sequence, is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector is suitable for driving expression of heterologous DNA in mammalian cells, particularly  
15 human cells. In one embodiment, the expression vector is p7313 (see figure 22).

In a further aspect the invention provides a plurality of particles, preferably gold particles, coated with DNA comprising one or more vectors encoding gp120 and nef and/or tat or nefTat. Preferably the particles are coated with a single vector which  
20 encodes gp120 and nef and tat, the latter most preferably in the form of a NefTat fusion protein. Most preferably one or more of the sequences are codon optimised for expression in human cells.

In a preferred aspect the DNA encoding the nef, tat and gp 120 is present on a single  
25 vector.

Preferably the vector comprises the nef, tat and gp 120 sequences inserted 3' to an enhanced HCMV IE1 promoter for efficient expression. This is preferably the HCMV immediate early promoter devoid of intron A, but including exon 1.

30 One suitable vector according to the invention is that denoted as p7313, further described below.

The vectors which comprise the nucleotide sequences described herein are  
35 administered in such amount as will be prophylactically or therapeutically effective. The quantity to be administered is generally in the range of one picogram to 1 milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery as

- 5 described herein. The exact quantity may vary depending on the weight of the patient being immunised and the precise route of administration.

Immunisations according to the invention may be performed with a combination of protein and DNA-based formulations. Adjuvanted protein vaccines induce mainly  
10 antibodies and T helper immune responses, while delivery of DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL) responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses. This is particularly relevant in the context of HIV, since both neutralising antibodies and CTL are thought to be important for the immune defence  
15 against HIV.

The DNA may be delivered as plasmid DNA or in the form of a recombinant live vector, e.g. a poxvirus vector or any other suitable live vector such as those described herein. Protein antigens may be injected once or several times followed by one or  
20 more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations.

A particular example of prime-boost immunisation according to the invention involves priming with DNA in the form of a recombinant live vector such as a  
25 modified poxvirus vector, for example Modified Virus Ankara (MVA) or a derivative thereof e.g. through passaging or genetic manipulation, or an alphavirus vector for example Venezuelan Equine Encephalitis Virus, followed by boosting with a protein, preferably an adjuvanted protein. Optionally the DNA is adjuvanted with a suitable DNA vaccine adjuvant known in the art.

30

The invention further provides a method of prophylactic or therapeutic immunisation which method comprises administering to a subject in need thereof a composition comprising

- a) an HIV Tat protein or polynucleotide; or
- 35 b) an HIV Nef protein or polynucleotide; or
- c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;

- 5 and an HIV gp120 protein or polynucleotide, in a prime-boost delivery wherein the protein or polynucleotide is delivered via a bombardment approach.

In a further aspect the invention provides a kit comprising at least two different vaccine compositions including a) a composition comprising a plurality of particles  
10 coated with DNA encoding gp120 and nef and/or tat or nefat as described herein and b) a composition comprising gp120 and nef and/or tat or nefat DNA or proteins as described herein wherein the DNA or proteins in b) are not coated onto particles.

The invention is illustrated in the accompanying examples and Figures:

15

## EXAMPLES

### General

20

The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs of these experiments since this gene is among those that are most closely related to the consensus Nef .

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment  
25 cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985.

30 The expression of the Nef and Tat genes could be in Pichia or any other host.

**Example 1. EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN PICHIA PASTORIS.**  
35

5 Nef protein, Tat protein and the fusion Nef-Tat were expressed in the methylotrophic yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1) promoter.

To express these HIV-1 genes a modified version of the integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues. This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent *AsuII* and *EcoRI* sites of PHIL-D2 vector (see Figure 2 which includes Seq ID Nos: 26 and 27). In addition to the His tail, this linker carries *NcoI*, *SpeI* and *XbaI* restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

#### 1.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14597 (encoding Nef-His protein), pRIT14598 (encoding Tat-His protein) and pRIT14599 (encoding fusion Nef-Tat-His).

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02.

25

*NcoI*

PRIMER 01 (Seq ID NO 1): 5' ATCGTCCATG.GGT.GGC.AAG.TGG.T 3'

*SpeI*

30 PRIMER 02 (Seq ID NO 2): 5' CGGCTACTAGTGCAGTTCTTGAA 3'

The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by *NcoI* and *SpeI*, purified on agarose gel and ligated to create the integrative plasmid pRIT14597 (see Figure 2).

35

The *tat* gene was amplified by PCR from a derivative of the pCV1 plasmid with primers 05 and 04:

5

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTCCTTCGGGCCT 3'

NcoI

10 PRIMER 05 (Seq ID NO 5): 5' ATCGTCCATGGAGCCAGTAGATC 3'

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose  
15 gel and ligated to create the integrative plasmid pRIT14598.

To construct pRIT14599, a 910bp DNA fragment corresponding to the *nef-tat*-His coding sequence was ligated between the EcoRI blunted(T4 polymerase) and NcoI sites of the PHIL-D2-MOD vector. The *nef-tat*-His coding fragment was  
20 obtained by XbaI blunted(T4 polymerase) and NcoI digestions of pRIT14596.

## 1.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-His, strain GS115 was transformed with linear NotI fragments carrying the respective  
25 expression cassettes plus the HIS4 gene to complement his4 in the host genome. Transformation of GS115 with NotI-linear fragments favors recombination at the AOXI locus.

30 Multicopy integrant clones were selected by quantitative dot blot analysis and the type of integration, insertion (Mut<sup>+</sup> phenotype) or transplacement (Mut<sup>s</sup> phenotype), was determined.

From each transformation, one transformant showing a high production level for the  
35 recombinant protein was selected :

Strain Y1738 (Mut<sup>+</sup> phenotype) producing the recombinant Nef-His protein,

5 a myristylated 215 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD  
vector

10 °205 a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

°A threonine and a serine created by the cloning procedure (cloning at SpeI  
site of PHIL-D2-MOD vector.

°One glycine and six histidines.

15 Strain Y1739 (Mut<sup>+</sup> phenotype) producing the Tat-His protein, a 95 amino acid  
protein which is composed of:

°A methionine created by the use of NcoI cloning site

°85 a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

20

°A threonine and a serine introduced by cloning procedure

°One glycine and six histidines

Strain Y1737(Mut<sup>s</sup> phenotype) producing the recombinant Nef-Tat-His fusion protein,  
25 a myristylated 302 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site

°205a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

30 °A threonine and a serine created by the cloning procedure

°85a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by the cloning procedure

°One glycine and six histidines

35

## **Example 2. EXPRESSION OF HIV-1 Tat-MUTANT IN PICHIA PASTORIS**



- 5 A mutant recombinant Tat protein has also been expressed. The mutant Tat protein must be **biologically inactive while maintaining its immunogenic epitopes.**

A double mutant *tat* gene, constructed by D.Clements (Tulane University) was selected for these constructs.

10

This *tat* gene (originates from BH10 molecular clone) bears **mutations in the active site region (Lys41→Ala) and in RGD motif (Arg78→Lys and Asp80→Glu)** (Virology 235: 48-64, 1997).

- 15 The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

## 2.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS

- 20 **pRIT14912(encoding Tat mutant-His protein) and pRIT14913(encoding fusion Nef-Tat mutant-His).**

The *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 05 and 04 (see section 1.1 construction of pRIT14598)

25

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14912

30

To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04.

SpeI

- 35 **PRIMER 03 (Seq ID NO 3): 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'**

SpeI

5 PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to  
10 create the integrative plasmid pRIT14913

## 2.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115.

Pichia pastoris strains expressing Tat mutant-His protein and the fusion Nef-Tat  
15 mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 1.2 .

Two recombinant strains producing Tat mutant-His protein ,a 95 amino-acids protein, were selected: Y1775 (Mut<sup>+</sup> phenotype) and Y1776(Mut<sup>s</sup> phenotype).  
20

One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut<sup>+</sup> phenotype).

25

### Example 3: FERMENTATION OF PICHIA PASTORIS PRODUCING RECOMBINANT TAT-HIS.

A typical process is described in the table hereafter.

30

Fermentation includes a growth phase (feeding with a glycerol-based medium according to an appropriate curve) leading to a high cell density culture and an induction phase (feeding with a methanol and a salts/micro-elements solution). During fermentation the growth is followed by taking samples and measuring their  
35 absorbance at 620 nm. During the induction phase methanol was added via a pump and its concentration monitored by Gas chromatography (on culture samples) and by on-line gas analysis with a Mass spectrometer. After fermentation the cells were

- 5 recovered by centrifugation at 5020g during 30' at 2-8°C and the cell paste stored at – 20°C. For further work cell paste was thawed, resuspended at an OD (at 620 nm) of 150 in a buffer (Na<sub>2</sub>HPO<sub>4</sub> pH7 50 mM, PMSF 5%, Isopropanol 4 mM) and disrupted by 4 passages in a DynoMill (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).
- 10 For evaluation of the expression samples were removed during the induction, disrupted and analyzed by SDS-Page or Western blot. On Coomassie blue stained SDS-gels the recombinant Tat-his was clearly identified as an intense band presenting a maximal intensity after around 72-96H induction.

5

Thawing of a Working seed vial	
↓	
Solid preculture 30°C, 14-16H	<u>Synthetic medium</u> : YNB + glucose + agar
↓	
Liquid preculture in two 2L erlenmeyer 30°C, 200 rpm	<u>Synthetic medium</u> : 2 x 400 ml YNB + glycerol Stop when OD > 1 (at 620 nm)
↓	
Inoculation of a 20L fermentor	5L initial medium (FSC006AA) 3 ml antifoam SAG471 (from Witco) Set-points: Temperature : 30°C Overpressure: 0.3 barg Air flow: 20 NI/min Dissolved O2: regulated > 40% pH : regulated at 5 by NH <sub>4</sub> OH
↓	
Fed-batch fermentation: growth phase Duration around 40H	Feeding with glycerol-based medium FFB005AA Final OD between 200-500 OD (620 nm)
Fed-batch fermentation: induction phase Duration: up to 97H	Feeding with methanol and with a salt/micro- elements solution (FSE021AB).
↓	
Centrifugation	5020g /30 min / 2-8°C
↓	
Recover cell paste and store at -20°C	
↓	
Thaw cells and resuspend at OD150 (620 nm) in buffer	<u>Buffer</u> : Na <sub>2</sub> HPO <sub>4</sub> pH7 50 mM, PMSF 5%, Isopropanol 4 mM
↓	
Cell disruption in Dyno-mill 4 passages	<u>Dyno-mill</u> : (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).
↓	
Transfer for extraction/purification	

5 **Media used for fermentation:****Solid preculture: (YNB + glucose + agar)**

Glucose:	10 g/l	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O:	0.0002 g/l	Acide folique:	
KH <sub>2</sub> PO <sub>4</sub> :	1 g/l	MnSO <sub>4</sub> .H <sub>2</sub> O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO <sub>4</sub> .7H <sub>2</sub> O:	0.5 g/l	H <sub>3</sub> BO <sub>3</sub> :	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl <sub>2</sub> .2H <sub>2</sub> O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl <sub>2</sub> .6H <sub>2</sub> O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl <sub>3</sub> .6H <sub>2</sub> O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO <sub>4</sub> .5H <sub>2</sub> O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	
ZnSO <sub>4</sub> .7H <sub>2</sub> O:	0.0004 g/l	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	5 g/l	Agar	18 g/l

**Liquid preculture ,(YNB + glycerol)**

Glycerol:	2% (v/v)	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O:	0.0002 g/l	Acide folique:	
KH <sub>2</sub> PO <sub>4</sub> :	1 g/l	MnSO <sub>4</sub> .H <sub>2</sub> O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO <sub>4</sub> .7H <sub>2</sub> O:	0.5 g/l	H <sub>3</sub> BO <sub>3</sub> :	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl <sub>2</sub> .2H <sub>2</sub> O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl <sub>2</sub> .6H <sub>2</sub> O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl <sub>3</sub> .6H <sub>2</sub> O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO <sub>4</sub> .5H <sub>2</sub> O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	
ZnSO <sub>4</sub> .7H <sub>2</sub> O:	0.0004 g/l	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	5 g/l		

**Initial fermentor charge: (FSC006AA)**

10	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	6.4 g/l	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O:	2.04 mg/l	
	KH <sub>2</sub> PO <sub>4</sub> :	9 g/l			
	MgSO <sub>4</sub> .7H <sub>2</sub> O:		4.7 g/l	MnSO <sub>4</sub> .H <sub>2</sub> O:	4.08
		mg/l			
15	CaCl <sub>2</sub> .2H <sub>2</sub> O:	0.94 g/l	H <sub>3</sub> BO <sub>3</sub> :	5.1 mg/l	
	FeCl <sub>3</sub> .6H <sub>2</sub> O:	10 mg/l	KI:	1.022 mg/l	
	HCl:	1.67 ml/l	CoCl <sub>2</sub> .6H <sub>2</sub> O:	0.91mg/l	
	CuSO <sub>4</sub> .5H <sub>2</sub> O:		0.408 mg/l	NaCl:	0.06
		g/l			
20	ZnSO <sub>4</sub> .7H <sub>2</sub> O:	4.08 mg/l	Biotine:	0.534 mg/l	

5

**Feeding solution used for growth phase (FFB005AA)**

	Glycérol:	38.7 % v/v	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O:	5.7
	mg/l			
10	MgSO <sub>4</sub> ·7H <sub>2</sub> O:	13 g/l	CuSO <sub>4</sub> ·5H <sub>2</sub> O:	1.13 mg/l
	CaCl <sub>2</sub> ·2H <sub>2</sub> O:	2.6 g/l	CoCl <sub>2</sub> ·6H <sub>2</sub> O:	2.5 mg/l
	FeCl <sub>3</sub> ·6H <sub>2</sub> O:	27.8mg/l	H <sub>3</sub> BO <sub>3</sub> :	14.2 mg/l
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	11.3 mg/l	Biotine:	1.5 mg/l
	MnSO <sub>4</sub> ·H <sub>2</sub> O:	11.3 mg/l	KI:	2.84mg/l
15	KH <sub>2</sub> PO <sub>4</sub> :	24.93 g/l	NaCl:	0.167 g/l

**Feeding solution of salts and micro-elements used during induction (FSE021AB):**

	KH <sub>2</sub> PO <sub>4</sub> :	45 g/l	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O:	10.2 mg/l	
20	MgSO <sub>4</sub> ·7H <sub>2</sub> O:		23.5 g/l	MnSO <sub>4</sub> ·H <sub>2</sub> O:	20.4
	mg/l				
	CaCl <sub>2</sub> ·2H <sub>2</sub> O:	4.70 g/l	H <sub>3</sub> BO <sub>3</sub> :	25.5 mg/l	
	NaCl:	0.3 g/l	KI:	5.11 mg/l	
	HCl:	8.3 ml/l	CoCl <sub>2</sub> ·6H <sub>2</sub> O:	4.55mg/l	
25	CuSO <sub>4</sub> ·5H <sub>2</sub> O:		2.04 mg/l	FeCl <sub>3</sub> ·6H <sub>2</sub> O:	50.0
	mg/l				
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O:	20.4 mg/l	Biotine:	2.70 mg/l	

30

**Example 4: PURIFICATION OF Nef-Tat-His FUSION PROTEIN (PICHIA PASTORIS)**

The purification scheme has been developed from 146g of recombinant *Pichia pastoris* cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps, Nef-Tat positive fractions are kept overnight in the cold room (+4°C); for longer time, samples are frozen at -20°C.

35

146g of *Pichia pastoris* cells

Homogenization

Buffer: 2L 50 mM PO<sub>4</sub> pH 7.0

final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation

JA10 rotor / 9500 rpm/ 30 min /  
room temperature

↓

Dyno-mill Pellet

↓

Wash

(1h - 4°C)

Buffer: +2L 10 mM PO<sub>4</sub> pH 7.5 -  
150mM - NaCl 0,5% empigen

↓

Centrifugation

JA10 rotor / 9500 rpm/ 30 min /  
room temperature

↓

Pellet

↓

Solubilisation

(O/N - 4°C)

Buffer: + 660ml 10 mM PO<sub>4</sub> pH  
7.5 - 150mM NaCl - 4.0M GuHCl

↓

Reduction

(4H – room temperature - in the dark)

+ 0,2M 2-mercaptoethanesulfonic  
acid, sodium salt (powder  
addition) / pH adjusted to 7.5  
(with 0,5M NaOH solution) before  
incubation

↓

reaction with iodoacetamide

(carbamidomethylation)

(1/2 h – room temperature - in the dark)

+ 0,25M Iodoacetamide (powder  
addition) / pH adjusted to 7.5  
(with 0,5M NaOH solution) before  
incubation

↓

Immobilized metal ion affinity

chromatography on Ni<sup>++</sup>-NTA-AgaroseEquilibration buffer: 10 mM PO<sub>4</sub>  
pH 7.5 - 150mM NaCl - 4.0M

(Qiagen - 30 ml of resin)

GuHCl

Washing buffer: 1) Equilibration  
buffer

2) 10 mM PO<sub>4</sub>

pH 7.5 - 150mM NaCl - 6M Urea

3) 10 mM PO<sub>4</sub>

pH 7.5 - 150mM NaCl - 6M Urea

- 25 mM Imidazol

Elution buffer: 10 mM PO<sub>4</sub> pH 7.5

- 150mM NaCl - 6M Urea - 0,5M

Imidazol

↓

Dilution

Down to an ionic strength of 18  
mS/cm<sup>2</sup>

Dilution buffer: 10 mM PO<sub>4</sub> pH  
7.5 - 6M Urea

↓

Cation exchange chromatography on SP  
Sephacrose FF

(Pharmacia - 30 ml of resin)

Equilibration buffer: 10 mM PO<sub>4</sub>

pH 7.5 - 150mM NaCl - 6.0M

Urea

Washing buffer: 1) Equilibration  
buffer

2) 10 mM PO<sub>4</sub>

pH 7.5 - 250mM NaCl - 6M Urea

Elution buffer: 10 mM Borate pH

9.0 - 2M NaCl - 6M Urea

↓

Concentration

up to 5 mg/ml

10kDa Omega membrane(Filtron)

↓

Gel filtration chromatography on  
Superdex200 XK 16/60

Elution buffer: 10 mM PO<sub>4</sub> pH 7.5

- 150mM NaCl - 6M Urea



(Pharmacia - 120 ml of resin)

5 ml of sample / injection → 5  
injections

↓

Dialysis

Buffer: 10 mM PO<sub>4</sub> pH 6.8 -

(O/N - 4°C)

150mM NaCl - 0,5M Arginin\*

↓

Sterile filtration

Millex GV 0,22µm

5

\* ratio: 0,5M Arginin for a protein concentration of 1600µg/ml.

### Purity

- 10 The level of purity as estimated by SDS-PAGE is shown in Figure 3 by Daiichi Silver Staining and in Figure 4 by Coomassie blue G250.

After Superdex200 step: > 95%

After dialysis and sterile filtration steps: > 95%

15

### Recovery

51mg of Nef-Tat-his protein are purified from 146g of recombinant Pichia pastoris cells (= 2L of Dyno-mill homogenate OD 55)

20

### Example 5: PURIFICATION OF OXIDIZED NEF-TAT-HIS FUSION PROTEIN IN PICHIA PASTORIS

- 25 The purification scheme has been developed from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps, Nef-Tat positive fractions are kept overnight in the cold room (+4°C); for longer time, samples are frozen at -20°C.

30

5

**73 g of Pichia pastoris cells****Homogenization**Buffer: 1L 50 mM PO<sub>4</sub> pH 7.0 –

Pefabloc 5 mM

final OD:50

**Dyno-mill disruption (4 passes)****Centrifugation**JA10 rotor / 9500 rpm/ 30 min / room  
temperature**Dyno-mill Pellet****Wash****(2h - 4°C)**Buffer: +1L 10 mM PO<sub>4</sub> pH 7.5 – 150  
mM NaCl - 0,5% Empigen**Centrifugation**JA10 rotor / 9500 rpm/ 30 min / room  
temperature**Pellet****Solubilisation****(O/N - 4°C)**Buffer: + 330ml 10 mM PO<sub>4</sub> pH 7.5 -  
150mM NaCl - 4.0M GuHCl**Immobilized metal ion affinity  
chromatography on Ni<sup>++</sup>-NTA-Agarose  
(Qiagen - 15 ml of resin)**Equilibration buffer: 10 mM PO<sub>4</sub> pH 7.5  
– 150 mM NaCl - 4.0 M GuHClWashing buffer: 1) Equilibration buffer  
2) 10 mM PO<sub>4</sub> pH 7.5  
– 150 mM NaCl – 6 M  
Urea3) 10 mM PO<sub>4</sub> pH 7.5

	–	150 mM NaCl – 6 M
	Urea -	25 mM Imidazol
	<u>Elution buffer:</u>	10 mM PO <sub>4</sub> pH 7.5 –
		150 mM NaCl – 6 M Urea - 0,5 M
		Imidazol
↓		
<b>Dilution</b>		Down to an ionic strength of 18 mS/cm <sup>2</sup>
	<u>Dilution buffer:</u>	10 mM PO <sub>4</sub> pH 7.5 – 6
		M Urea
↓		
<b>Cation exchange chromatography on SP</b>	<u>Equilibration buffer:</u>	10 mM PO <sub>4</sub> pH
<b>Sepharose FF</b>		7.5 – 150 mM NaCl - 6.0 M Urea
<b>(Pharmacia - 7 ml of resin)</b>	<u>Washing buffer:</u>	1) Equilibration buffer
		2) 10 mM PO <sub>4</sub> pH 7.5
	–	250 mM NaCl – 6 M
		Urea
	<u>Elution buffer:</u>	10 mM Borate pH 9.0 –
		2 M NaCl – 6 M Urea
↓		
<b>Concentration</b>		up to 0,8 mg/ml
		10kDa Omega membrane(Filtron)
↓		
<b>Dialysis</b>	<u>Buffer:</u>	10 mM PO <sub>4</sub> pH 6.8 – 150 mM
<b>(O/N - 4°C)</b>		NaCl – 0,5 M Arginin
↓		
<b>Sterile filtration</b>		Millex GV 0,22µm

5

→ Level of purity estimated by SDS-PAGE is shown in Figure 6 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

10 After dialysis and sterile filtration steps: > 95%

5

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

2,8 mg of oxidized Nef-Tat-his protein are purified from 73 g of recombinant *Pichia pastoris* cells (wet weight) or 1 L of Dyno-mill homogenate OD 50.

10

### **Example 6: PURIFICATION OF REDUCED TAT-HIS PROTEIN (PICHIA PASTORIS)**

15

The purification scheme has been developed from 160 g of recombinant *Pichia pastoris* cells (wet weight) or 2L Dyno-mill homogenate OD 66. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

20

160 g of *Pichia pastoris* cells

↓

Homogenization

Buffer: +2 L 50 mM PO<sub>4</sub> pH 7.0 – 4 mM PMSF  
final OD:66

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation

JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Dyno-mill Pellet

↓

Wash

Buffer: +2 L 10 mM PO<sub>4</sub> pH 7.5 – 150 mM NaCl  
- 1% Empigen

(1h - 4°C)

↓

Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4°C)	<u>Buffer</u> : + 660 ml 10 mM PO <sub>4</sub> pH 7.5 – 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Reduction (4H - room temperature - in the dark)	+ 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
reaction with iodoacetamide (1/2 h - room temperature - in the dark)	+ 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni <sup>++</sup> -NTA-Agarose (Qiagen - 60 ml of resin)	<u>Equilibration buffer</u> : 10 mM PO <sub>4</sub> pH 7.5 – 150 mM NaCl - 4.0 M GuHCl <u>Washing buffer</u> : 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO <sub>4</sub> pH 7.5 – 150 mM NaCl - 6M Urea - 35 mM Imidazol <u>Elution buffer</u> : 10 mM PO <sub>4</sub> pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm <u>Dilution buffer</u> : 20 mM Borate pH 8.5 – 6 M Urea
↓	
Cation exchange chromatography on SP	<u>Equilibration buffer</u> : 20 mM Borate pH 8.5 -

Sepharose FF

150 mM NaCl - 6.0 M Urea

(Pharmacia - 30 ml of resin)

Washing buffer: Equilibration bufferElution buffer: 20 mM Borate pH 8.5 – 400 mM  
NaCl - 6.0 M Urea

↓

Concentration

up to 1,5 mg/ml

10kDa Omega membrane(Filtron)

↓

Dialysis

Buffer: 10 mM PO<sub>4</sub> pH 6.8 – 150 mM NaCl -  
0,5 M Arginin

↓

Sterile filtration

Millex GV 0,22 µm

5

- 10 → Level of purity estimated by SDS-PAGE as shown in Figure 7(Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

- 15 → Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

48 mg of reduced Tat-his protein are purified from 160 g of recombinant

Pichia

pastoris cells (wet weight) or 2 L of

Dyno-mill homogenate OD 66.

20

### Example 7: Purification of oxidized Tat-his protein (Pichia Pastoris)

25

- 5 The purification scheme has been developed from 74 g of recombinant *Pichia pastoris* cells (wet weight) or 1L Dyno-mill homogenate OD60. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

74 g of *Pichia pastoris* cells

↓

Homogenization

Buffer: +1 L 50 mM PO<sub>4</sub> pH 7.0 – 5 mM Pefabloc  
final OD:60

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation

JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Dyno-mill Pellet

↓

Wash

Buffer: +1 L 10 mM PO<sub>4</sub> pH 7.5 – 150 mM NaCl  
- 1% Empigen

(1h - 4°C)

↓

Centrifugation

JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Pellet

↓

Solubilisation

Buffer: + 330 ml 10 mM PO<sub>4</sub> pH 7.5 – 150 mM  
NaCl - 4.0 M GuHCl

(O/N - 4°C)

↓

Centrifugation

JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Immobilized metal ion affinity

Equilibration buffer: 10 mM PO<sub>4</sub> pH 7.5 – 150 mM

chromatography on  $\text{Ni}^{++}$ -NTA-Agarose

NaCl - 4.0 M GuHCl

(Qiagen - 30 ml of resin)

Washing buffer: 1) Equilibration buffer

2) 10 mM  $\text{PO}_4$  pH 7.5 – 150 mM

NaCl – 6 M Urea

3) 10 mM  $\text{PO}_4$  pH 7.5 – 150 mM

NaCl – 6 M Urea - 35 mM

Imidazol

Elution buffer: 10 mM  $\text{PO}_4$  pH 7.5 – 150 mM

NaCl – 6 M Urea - 0,5 M Imidazol

↓

Dilution

Down to an ionic strength of 12 mS/cm

Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea

↓

Cation exchange chromatography on SP

Equilibration buffer: 20 mM Borate pH 8.5 -

150 mM NaCl - 6.0 M Urea

Sepharose FF

Washing buffer: 1) Equilibration buffer

2) 20 mM Borate pH 8.5 -

400 mM NaCl - 6.0 M Urea

(Pharmacia - 15 ml of resin)

Elution buffer: 20 mM Piperazine pH 11.0 – 2 M

NaCl – 6 M Urea

↓

Concentration

up to 1,5 mg/ml

10 kDa Omega membrane(Filtron)

↓

Dialysis

Buffer: 10 mM  $\text{PO}_4$  pH 6.8 – 150 mM NaCl -

(O/N - 4°C)

0,5 M Arginin

↓

Sterile filtration

Millex GV 0,22  $\mu\text{m}$

5

→ Level of purity estimated by SDS-PAGE as shown in Figure 8 (Daichi Silver Staining, Coomassie blue G250, Western blotting):



5 After dialysis and sterile filtration steps: > 95%

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

10 19 mg of oxidized Tat-his protein are purified from 74 g of recombinant  
Pichia pastoris cells (wet weight) or 1 L of  
Dyno-mill homogenate OD 60.

**Example 8: PURIFICATION OF SIV REDUCED NEF-HIS PROTEIN  
(PICHIA PASTORIS)**

20 The purification scheme has been developed from 340 g of recombinant Pichia  
pastoris cells (wet weight) or 4 L Dyno-mill homogenate OD 100. The  
chromatographic steps are performed at room temperature. Between steps, Nef  
positive fractions are kept overnight in the cold room (+4°C); for longer time,  
samples are frozen at -20°C.

25

**340 g of Pichia pastoris cells**

↓

**Homogenization**

Buffer: 4L 50 mM PO<sub>4</sub> pH 7.0 – PMSF

4 mM

final OD:100

↓

**Dyno-mill disruption (4 passes)**

↓

**Centrifugation**

JA10 rotor / 9500 rpm/ 60 min / room  
temperature

↓

**Dyno-mill Pellet****Solubilisation****(O/N - 4°C)****Centrifugation****Reduction****(4H - room temperature - in the dark)****Reaction with iodoacetamide****(1/2 h - room temperature - in the dark)**

**Immobilized metal ion affinity  
chromatography on Ni<sup>++</sup>-NTA-Agarose  
(Qiagen - 40 ml of resin)**

**Concentration**

**Gel filtration chromatography on  
Superdex 200**

Buffer: + 2,6 L 10 mM PO<sub>4</sub> pH 7.5 -  
150mM NaCl - 4.0M GuHCl

JA10 rotor / 9500 rpm / 30 min / room  
temperature

+ 0,2 M 2-mercaptoethanesulfonic acid,  
sodium salt (powder addition) / pH  
adjusted to 7.5 (with 1 M NaOH  
solution) before incubation

+ 0,25 M Iodoacetamide (powder  
addition) / pH adjusted to 7.5 (with 1  
M NaOH solution) before incubation

Equilibration buffer: 10 mM PO<sub>4</sub> pH 7.5  
– 150 mM NaCl - 4.0 M GuHCl

Washing buffer: 1) Equilibration buffer  
2) 10 mM PO<sub>4</sub> pH 7.5  
– 150 mM NaCl – 6 M  
Urea - 25 mM Imidazol

Elution buffer: 10 mM PO<sub>4</sub> pH 7.5 –  
150 mM NaCl – 6 M Urea - 0,5 M  
Imidazol

up to 3 mg/ml  
10kDa Omega membrane(Filtron)

Elution buffer: 10 mM PO<sub>4</sub> pH 7.5 –  
150 mM NaCl – 6 M Urea

(Pharmacia - 120 ml of resin)



**Concentration**

up to 1,5 mg/ml

10kDa Omega membrane(Filtron)



**Dialysis**

Buffer: 10 mM PO<sub>4</sub> pH 6.8 – 150 mM

(O/N - 4°C)

NaCl – Empigen 0,3%



**Sterile filtration**

Millex GV 0,22µm

5

→ Level of purity estimated by SDS-PAGE as shown in Figure 9 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

10

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of SIV reduced Nef -his protein are purified from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L of Dyno-mill homogenate OD 100.

15

### **Example 9: PURIFICATION OF HIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)**

20

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps, Nef positive fractions are kept overnight in the cold room (+4°C); for longer time, samples are frozen at -20°C.

25

5

**160 g of Pichia pastoris cells****Homogenization**Buffer: 3 L 50 mM PO<sub>4</sub> pH 7.0 –

Pefabloc 5 mM final OD:50

**Dyno-mill disruption (4 passes)****Freezing/Thawing****Centrifugation**JA10 rotor / 9500 rpm/ 60 min / room  
temperature**Dyno-mill Pellet****Solubilisation**Buffer: + 1 L 10 mM PO<sub>4</sub> pH 7.5 -**(O/N - 4°C)**

150mM NaCl - 4.0M GuHCl

**Centrifugation**JA10 rotor / 9500 rpm / 60 min / room  
temperature**Reduction****(3 h - room temperature - in the dark)**+ 0,1 M 2-mercaptoethanesulfonic acid,  
sodium salt (powder addition) / pH  
adjusted to 7.5 (with 1 M NaOH  
solution) before incubation**Reaction with iodoacetamide****(1/2 h - room temperature - in the dark)**+ 0,15 M Iodoacetamide (powder  
addition) / pH adjusted to 7.5 (with 1  
M NaOH solution) before incubation**Immobilized metal ion affinity**Equilibration buffer: 10 mM PO<sub>4</sub> pH 7.5

**chromatography on Ni<sup>++</sup>-NTA-Agarose**  
**(Qiagen - 10 ml of resin)**

-- 150 mM NaCl - 4.0 M GuHCl

Washing buffer: 1) Equilibration buffer

2) 10 mM PO<sub>4</sub> pH 7.5

-- 150 mM NaCl -- 6 M

Urea

3) 10 mM PO<sub>4</sub> pH 7.5

-- 150 mM NaCl -- 6 M

Urea - 25 mM Imidazol

Elution buffer: 10 mM Citrate pH 6.0 -

150 mM NaCl -- 6 M Urea - 0,5 M

Imidazol

↓

**Concentration**

up to 3 mg/ml

10kDa Omega membrane(Filtron)

↓

**Gel filtration chromatography on**  
**Superdex 200**

Elution buffer: 10 mM PO<sub>4</sub> pH 7.5 --

150 mM NaCl -- 6 M Urea

**(Pharmacia - 120 ml of resin)**

↓

**Dialysis**

Buffer: 10 mM PO<sub>4</sub> pH 6.8 -- 150 mM

**(O/N - 4°C)**

NaCl -- 0,5M Arginin

↓

**Sterile filtration**

Millex GV 0,22µm

5

→ Level of purity estimated by SDS-PAGE as shown in Figure 10 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

10

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

- 5 20 mg of HIV reduced Nef -his protein are purified from 160 g of recombinant *Pichia pastoris* cells (wet weight) or 3 L of Dyno-mill homogenate OD 50.

### Example 10: EXPRESSION OF SIV *nef* SEQUENCE IN *PICHLA PASTORIS*

10

In order to evaluate Nef and Tat antigens in the pathogenic SHIV challenge model, we have expressed the Nef protein of simian immunodeficiency virus (SIV) of macaques, SIVmac239 (Aids Research and Human Retroviruses, 6:1221-1231,1990).

- 15 In the Nef coding region, SIV mac 239 has an in-frame stop codon after 92aa predicting a truncated product of only 10kD. The remainder of the Nef reading frame is open and would be predicted to encode a protein of 263aa (30kD) in its fully open form.

- 20 Our starting material for SIVmac239 *nef* gene was a DNA fragment corresponding to the complete coding sequence, cloned on the LX5N plasmid (received from Dr R.C. Desrosiers, Southborough,MA,USA).

This SIV *nef* gene is mutated at the premature stop codon (nucleotide G at position 9353 replaces the original T nucleotide) in order to express the full-length SIVmac239 Nef protein.

25

- To express this SIV *nef* gene in *Pichia pastoris*, the PHIL-D2-MOD Vector (previously used for the expression of HIV-1 *nef* and *tat* sequences) was used. The recombinant protein is expressed under the control of the inducible alcohol oxidase (AOX1) promoter and the c-terminus of the protein is elongated by a  
30 Histidine affinity tail that will facilitate the purification.

### 10.1 CONSTRUCTION OF THE INTEGRATIVE VECTOR pRIT 14908

35

To construct pRIT 14908, the SIV *nef* gene was amplified by PCR from the pLX5N/SIV-NEF plasmid with primers SNEF1 and SNEF2.

5

PRIMER SNEF1: 5' ATCGTCCATG.GGTGGAGCTATTTT 3'

NcoI

(Seq ID No: 6)

10

PRIMER SNEF2: 5' CGGCTACTAGTGCGAGTTTCCTT 3'

SpeI

(Seq ID No: 7)

- 15 The SIV *nef* DNA region amplified starts at nucleotide 9077 and terminates at nucleotide 9865 ( Aids Research and Human Retroviruses, 6:1221-1231,1990).

An NcoI restriction site (with carries the ATG codon of the *nef* gene) was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end.

- 20 The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI. Since one NcoI restriction site is present on the SIV *nef* amplified sequence (at position 9286), two fragments of respectively  $\pm 200$ bp and  $\pm 600$ bp were obtained, purified on agarose gel and ligated to PHIL-D2-MOD vector. The resulting recombinant plasmid received, after verification of the *nef* amplified  
25 region by automated sequencing, the pRIT 14908 denomination.

## 10.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

- 30 To obtain *Pichia pastoris* strain expressing SIV *nef*-His, strain GS115 was transformed with a linear NotI fragment carrying only the expression cassette and the HIS4 gene (Fig.11).

This linear NotI DNA fragment ,with homologies at both ends with AOX1 resident *P.pastoris* gene, favors recombination at the AOX1 locus.

- 35 Multicopy integrant clones were selected by quantitative dot blot analysis .

One transformant showing the best production level for the recombinant protein was selected and received the Y1772 denomination.

5

Strain Y1772 produces the recombinant SIV Nef-His protein, a **272 amino acids** protein which would be composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector .

10 °262 amino acids (aa) of Nef protein (starting at aa 2 and extending to aa 263, see Figure 12)

°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector (Fig.11 – includes Seq ID No: 29).

°One glycine and six histidines.

15

Nucleic and Protein sequences are shown in Figure 12 (Seq ID Nos: 30 and 31).

### 10.3 CHARACTERIZATION OF THE EXPRESSED PRODUCT OF STRAIN

20 Y1772.

#### Expression level

After 16 hours induction in medium containing 1% methanol as carbon source,  
25 abundance of the recombinant Nef-His protein, was estimated at **10%** of total protein (Fig.13, lanes 3-4).

#### Solubility

30 Induced cultures of recombinant strain Y1772 producing the Nef-His protein were centrifuged. Cell pellets were resuspended in breaking buffer, disrupted with 0.5mm glass beads and the cell extracts were centrifuged. The proteins contained in the insoluble pellet (P) and in the soluble supernatant (S) were compared on a Coomassie Blue stained SDS-PAGE10%.

35 As shown in figure 13, the majority of the recombinant protein from strain Y1772 (lanes 3-4) is associated with the insoluble fraction.



- 5 Strain Y1772 which presents a satisfactory recombinant protein expression level is used for the production and purification of SIV Nef-His protein.

### Example 11: EXPRESSION OF GP120 IN CHO

10

A stable CHO-K1 cell line which produces a recombinant gP120 glycoprotein has been established. Recombinant gP120 glycoprotein is a recombinant truncated form of the gP120 envelope protein of HIV-1 isolate W61D. The protein is excreted into the cell culture medium, from which it is subsequently purified.

15

#### Construction of gp120 transfection plasmid pRIT13968

The envelope DNA coding sequence (including the 5' exon of tat and rev) of HIV-1 isolate W61D was obtained (Dr. Tersmette, CCB, Amsterdam) as a genomic gp160 envelope containing plasmid W61D (Nco-XhoI). The plasmid was designated  
20 pRIT13965.

In order to construct a gp120 expression cassette a stop codon had to be inserted at the amino acid glu 515 codon of the gp160 encoding sequence in pRIT13965 using a primer oligonucleotide sequence (DIR 131) and PCR technology. Primer DIR 131  
25 contains three stop codons (in all open reading frames) and a SalI restriction site.

The complete gp120 envelope sequence was then reconstituted from the N-terminal BamHI-DraI fragment (170 bp) of a gp160 plasmid subclone pW61d env (pRIT13966) derived from pRIT13965, and the DraI-SalI fragment (510 bp) generated  
30 by PCR from pRIT13965. Both fragments were gel purified and ligated together into the E.coli plasmid pUC18, cut first by SalI (klenow treated), and then by BamHI. This resulted in plasmid pRIT13967. The gene sequence of the XmaI-SalI fragment (1580 bp) containing the gp120 coding cassette was sequenced and found to be identical to the predicted sequence. Plasmid RIT13967 was ligated into the CHO GS-expression  
35 vector pEE14 (Celltech Ltd., UK) by cutting first with BclI (klenow treated) and then by XmaI. The resulting plasmid was designated pRIT13968.

### 5 Preparation of Master Cell Bank

The gp120-construct (pRIT13968) was transfected into CHO cells by the classical CaPO<sub>4</sub>-precipitation/glycerol shock procedure. Two days later the CHOK1 cells were subjected to selective growth medium (GMEM + methionine sulfoximine (MSX) 25 µM + Glutamate + asparagine + 10% Foetal calf serum ). Three chosen transfectant  
10 clones were further amplified in 175m<sup>2</sup> flasks and few cell vials were stored at -80°C. C-env 23,9 was selected for further expansion.

A small prebank of cells was prepared and 20 ampoules were frozen. For preparation of the prebank and the MCB, cells were grown in GMEM culture medium,  
15 supplemented with 7.5 % fetal calf serum and containing 50 µM MSX. These cell cultures were tested for sterility and mycoplasma and proved to be negative.

The Master Cell Bank CHOK1 env 23.9 (at passage 12) was prepared using cells derived from the premaster cell bank. Briefly, two ampoules of the premaster seed  
20 were seeded in medium supplemented with 7.5% dialysed foetal bovine serum. The cells were distributed in four culture flasks and cultured at 37°C. After cell attachment the culture medium was changed with fresh medium supplemented with 50 µM MSX. At confluence, cells were collected by trypsination and subcultured with a 1/8 split ratio in T-flasks - roller bottle - cell factory units. Cells were collected from  
25 cell factory units by trypsination and centrifugation. The cell pellet was resuspended in culture medium supplemented with DMSO as cryogenic preservative. Ampoules were prelabelled, autoclaved and heat-sealed (250 vials). They were checked for leaks and stored overnight at -70°C before storage in liquid nitrogen.

### 30 Cell Culture And Production Of Crude Harvest

Two vials from a master cell bank are thawed rapidly. Cells are pooled and inoculated in two T-flasks at 37° ± 1°C with an appropriate culture medium supplemented with 7.5 % dialysed foetal bovine (FBS) serum. When reaching confluence (passage 13), cells are collected by trypsinisation, pooled and expanded in 10 T-flasks as above.  
35 Confluent cells (passage 14) are trypsinised and expanded serially in 2 cell factory units (each 6000 cm<sup>2</sup>; passage 15), then in 10 cell factories (passage 16). The growth culture medium is supplemented with 7.5 % dialysed foetal bovine (FBS) serum and

- 5 1% MSX. When cells reach confluence, the growth culture medium is discarded and replaced by "production medium" containing only 1 % dialysed foetal bovine serum and no MSX. Supernatant is collected every two days (48 hrs-interval) for up to 32 days. The harvested culture fluids are clarified immediately through a 1.2-0.22 µm filter unit and kept at -20°C before purification.

10

### **Example 12: PURIFICATION OF HIV GP 120 (W61D CHO) FROM CELL CULTURE FLUID**

- All purification steps are performed in a cold room at 2-8°C. pH of buffers are  
15 adjusted at this temperature and are filtered on 0.2 µm filter. They are tested for pyrogen content (LAL assay). Optical density at 280 nm, pH and conductivity of column eluates are continuously monitored.

#### **(i) Clarified Culture Fluid**

- 20 The harvested clarified cell culture fluid (CCF) is filter-sterilized and Tris buffer, pH 8.0 is added to 30 mM final concentration. CCF is stored frozen at -20°C until purification.

#### **(ii) Hydrophobic Interaction Chromatography**

- 25 After thawing, ammonium sulphate is added to the clarified culture fluid up to 1 M. The solution is passed overnight on a TSK/TOYOPEARL-BUTYL 650 M (TOSHAAS) column, equilibrated in 30 mM Tris buffer- pH 8.0 - 1 M ammonium sulphate. Under these conditions, the antigen binds to the gel matrix. The column is washed with a decreasing stepwise ammonium sulphate gradient. The antigen is  
30 eluted at 30 mM Tris buffer- pH 8.0 - 0.25 M ammonium sulphate.

#### **(iii) Anion-exchange Chromatography**

- After reducing the conductivity of the solution between 5 and 6 mS/cm, the gP120 pool of fractions is loaded onto a Q-sepharose Fast Flow (Pharmacia) column,  
35 equilibrated in Tris-saline buffer - pH 8.0. The column is operated on a negative mode, i.e. gP120 does not bind to the gel, while most of the impurities are retained.

5      (iv)      Concentration and diafiltration by ultrafiltration

In order to increase the protein concentration, the gP120 pool is loaded on a FILTRON membrane "Omega Screen Channel", with a 50 kDa cut-off. At the end of the concentration, the buffer is exchanged by diafiltration with 5 mM phosphate buffer containing  $\text{CaCl}_2$  0.3 mM, pH 7.0. If further processing is not performed

10 immediately, the gP120 pool is stored frozen at  $-20^\circ\text{C}$ . After thawing the solution is filtered onto a 0.2  $\mu\text{M}$  membrane in order to remove insoluble materiel.

(v)      Chromatography on hydroxyapatite

The gP120 UF pool is loaded onto a macro-Prep Ceramic Hydroxyapatite, type II  
15 (Biorad) column equilibrated in 5 mM phosphate buffer +  $\text{CaCl}_2$  0.3 mM, pH 7.0.

The column is washed with the same buffer. The antigen passes through the column and impurities bind to the column.

(vi)      Cation exchange chromatography

20 The gP120 pool is loaded on a CM/TOYOPEARL-650 S (TOSHAAS) column equilibrated in acetate buffer 20 mM, pH 5.0. The column is washed with the same buffer, then acetate 20 mM, pH 5.0 and NaCl 10 mM. The antigen is then eluted by the same buffer containing 80 mM NaCl.

25 (vii)      Ultrafiltration

In order to augment the virus clearance capacity of the purification process, an additional ultrafiltration step is carried out. The gP120 pool is subjected to ultrafiltration onto a FILTRON membrane "Omega Screen Channel", cut-off 150 kDa. This pore-size membrane does not retain the antigen. After the process, the diluted  
30 antigen is concentrated on the same type of membrane (Filtron) but with a cut-off of 50 kDa.

(viii)      Size exclusion Gel Chromatography

The gP120 pool is applied to a SUPERDEX 200 (PHARMACIA) column in order to  
35 exchange the buffer and to eliminate residual contaminants. The column is eluted with phosphate buffer saline (PBS).

5

(ix) Sterile filtration and storage

Fractions are sterilized by filtration on a 0.2  $\mu$ M PVDF membrane (Millipore). After sterile filtration, the purified bulk is stored frozen at -20°C up to formulation. The purification scheme is summarized by the flow sheet below.

10

⇒ Level of purity of the purified bulk estimated by SDS-PAGE analysis

(Silver staining / Coomassie Blue / Western Blotting) is  $\geq 95\%$ .

⇒ Production yield is around 2.5 mg /L CCF (according to Lowry assay) -

Global purification yield is around 25% (according to Elisa assay)

15

⇒ Purified material is stable 1 week at 37°C (according to WB analysis)

Purification of gp120 from culture fluid

20 Mark  $\checkmark$  indicate steps that are critical for virus removal.

CLARIFIED CULTURE FLUID

↓

HYDROPHOBIC INTERACTION CHROMATOGRAPHY  
(BUTYL -TOYOPEARL 650 M)

↓

ANION EXCHANGE CHROMATOGRAPHY (NEGATIVE  
MODE)

$\checkmark$

(Q-SEPHAROSE)

↓

50 KD ULTRAFILTRATION

(CONCENTRATION AND BUFFER EXCHANGE)

↓

(STORAGE -20°C)



HYDROXYAPATITE CHROMATOGRAPHY (NEGATIVE  
MODE)

(MACROPREP CERAMIC HYDROXYAPATITE II)



CATION EXCHANGE CHROMATOGRAPHY

(CM-TOYOPEARL 650 S)



150 KD ULTRAFILTRATION

√

(OMEGA MEMBRANES / FILTRON)



50 KD ULTRAFILTRATION

(CONCENTRATION)



SIZE EXCLUSION CHROMATOGRAPHY

√

(SUPERDEX 200)

STERILE FILTRATION



**PURIFIED BULK**

STORAGE -20°C

### 5 **Example 13: VACCINE PREPARATION**

A vaccine prepared in accordance with the invention comprises the expression products of one or more DNA recombinants encoding an antigen. Furthermore, the formulations comprise a mixture of 3 de -O-acylated monophosphoryl lipid A 3D-MPL and QS21 in an oil/water emulsion or an oligonucleotide containing  
10 unmethylated CpG dinucleotide motifs and aluminium hydroxide as carrier.

**3D-MPL:** is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria *Salmonella minnesota*.

15

Experiments have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral immunity and a  $T_{HI}$  type of cellular immunity.

**QS21:** is a saponin purified from a crude extract of the bark of the *Quillaja Saponaria*  
20 *Molina* tree, which has a strong adjuvant activity: it induces both antigen-specific lymphoproliferation and CTLs to several antigens.

Experiments have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and  $T_{HI}$  type cellular immune responses.

25 **The oil/water emulsion** is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprises 5% squalene, 5% tocopherol, 2% Tween 80 and has an average particle size of 180 nm (see WO 95/17210).

30 Experiments performed have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

#### **Preparation of the oil/water emulsion (2 fold concentrate)**

35 Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is

- 5 added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

#### **Preparation of oil in water formulation.**

- 10 Antigens (100 µg gp120, 20 µg NefTat, and 20 µg SIV Nef, alone or in combination) were diluted in 10 fold concentrated PBS pH 6.8 and H<sub>2</sub>O before consecutive addition of the oil in water emulsion, 3D-MPL (50µg), QS21 (50µg) and 1 µg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (250µl for a dose of 500µl).

15

All incubations were carried out at room temperature with agitation.

- CpG oligonucleotide (CpG) is a synthetic unmethylated oligonucleotide containing one or several CpG sequence motifs. CpG is a very potent inducer of T<sub>H1</sub> type immunity compared to the oil in water formulation that induces mainly a mixed T<sub>H1</sub>/T<sub>H2</sub> response. CpG induces lower level of antibodies than the oil in water formulation and a good cell mediated immune response. CpG is expected to induce lower local reactogenicity.
- 20

- 25 Preparation of CpG oligonucleotide solution: CpG dry powder is dissolved in H<sub>2</sub>O to give a solution of 5 mg/ml CpG.

#### **Preparation of CpG formulation.**

- The 3 antigens were dialyzed against NaCl 150 mM to eliminate the phosphate ions that inhibit the adsorption of gp120 on aluminium hydroxide.
- 30 The antigens diluted in H<sub>2</sub>O (100 µg gp120, 20 µg NefTat and 20 µg SIV Nef) were incubated with the CpG solution (500 µg CpG) for 30 min before adsorption on Al(OH)<sub>3</sub> to favor a potential interaction between the His tail of NefTat and Nef antigens and the oligonucleotide (stronger immunostimulatory effect of CpG described when bound to the antigen compared to free CpG). Then were consecutively added at 5 min interval Al(OH)<sub>3</sub> (500 µg), 10 fold concentrated NaCl and 1 µg/ml thiomersal as preservative.
- 35



5

All incubations were carried out at room temperature with agitation.

# **Example 14: IMMUNIZATION AND SHIV CHALLENGE EXPERIMENT IN RHESUS MONKEYS.**

## **First Study**

Groups of 4 rhesus monkeys were immunized intramuscularly at 0, 1 and 3 months with the following vaccine compositions:

Group 1:	Adjuvant 2	+ gp120		
Group 2:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 3:	Adjuvant 2		+ NefTat*	+ SIV Nef
Group 4:	Adjuvant 6	+ gp120	+ NefTat	+ SIV Nef
Group 5:	Adjuvant 2		+ NefTat	+ SIV Nef
Group 6:	Adjuvant 2			

Adjuvant 2 comprises squalene/tocopherol/Tween 80/3D-MPL/QS21 and Adjuvant 6 comprises alum and CpG.

Tat\* represents mutated Tat, in which Lys41→Ala and in RGD motif Arg78→Lys and Asp80→Glu ( Virology 235: 48-64, 1997).

One month after the last immunization all animals were challenged with a pathogenic SHIV (strain 89.6p). From the week of challenge (wk16) blood samples were taken periodically at the indicated time points to determine the % of CD4-positive cells among peripheral blood mononuclear cells by FACS analysis (Figure 14) and the concentration of RNA viral genomes in the plasma by bDNA assay (Figure 15).

## **Results**

5 All animals become infected after challenge with SHIV<sub>89.6p</sub>.

CD4-positive cells decline after challenge in all animals of groups 1, 3, 5 and 6 except one animal in each of groups 1 and 6 (control group). All animals in group 2 exhibit a slight decrease in CD4-positive cells and recover to baseline levels over time. A

10 similartrend is observed in group 4 animals (Figure 14).

Virus load data are almost the inverse of CD4 data. Virus load declines below the level of detection in ¾ group 2 animals (and in the one control animal that maintains its CD4-positive cells), and the fourth animal shows only marginal virus load. Most  
15 of the other animals maintain a high or intermediate virus load (Figure 15).

Surprisingly, anti-Tat and anti-Nef antibody titres measured by ELISA were 2 to 3-fold higher in Group 3 (with mutated Tat) than in Group 5 (the equivalent Group with non-mutated Tat) throughout the course of the study.

20

At week 68 (56 weeks post challenge) all animals from the groups that had received the full antigen combination (groups 2 and 4) were still alive, while most of the animals in the other groupshad to be euthanized due to AIDS-like symptoms. The surviving animals per group were:

25

Group 1: 2/4

Group 2: 4/4

Group 3: 0/4

Group 4 4/4

30 Group 5 0/4

Group 6 1/4

## Conclusions

35

The combination of gp120 and NefTat (in the presence of SIV Nef) prevents the loss of CD4-positive cells, reduces the virus load in animals infected with pathogenic

- 5 SHIV<sub>89.6p</sub>, and delays or prevents the development of AIDS-like disease symptoms, while gp120 or NefTat/SIV Nef alone do not protect from the pathologic consequences of the SHIV challenge.

- 10 The adjuvant 2 which is an oil in water emulsion comprising squalene, tocopherol and Tween 80, together with 3D-MPL and QS21 seems to have a stronger effect on the study endpoints than the alum / CpG adjuvant.

### **Second study**

- 15 A second rhesus monkey SHIV challenge study was conducted to confirm the efficacy of the candidate vaccine gp120/NefTat + adjuvant and to compare different Tat-based antigens. The study was conducted by a different laboratory.

The design of the study was as follows.

20

Groups of 6 rhesus monkeys were immunized at 0, 4 and 12 weeks with injections i.m. and challenged at week 16 with a standard dose of pathogenic SHIV<sub>89.6p</sub>.

Group 1 is the repeat of Group 2 in the first study.

25

Group 1:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 2:	Adjuvant 2	+ gp120	+ Tat (oxidised)	
Group 3:	Adjuvant 2	+ gp120	+ Tat (reduced)	
Group 4	Adjuvant 2			

30

The follow-up/endpoints were again % CD4-positive cells, virus load by RT-PCR, morbidity and mortality

### **Results**

35

All animals except one in group 2 become infected after challenge with SHIV<sub>89.6p</sub>.

5 CD4-positive cells decline significantly after challenge in all animals of control group 4 and group 3, and in all but one animals of group 2. Only one animal in group 1 shows a marked decrease in CD4-positive cells. Unlike the animals from the first study, the monkeys in the second experiment display a stabilisation of CD4-positive cells at different levels one month after virus challenge (Figure 16). The stabilisation  
10 is generally lower than the initial % of CD4-positive cells, but will never lead to a complete loss of the cells. This may be indicative of a lower susceptibility to SHIV-induced disease in the monkey population that was used for the second study. Nonetheless, a beneficial effect of the gp120/NefTat/SIV Nef vaccine and the two gp120/Tat vaccines is demonstrable. The number of animals with a % of CD4-positive  
15 cells above 20 is 5 for the vaccinated animals, while none of the control animals from the adjuvant group remains above that level.

Analysis of RNA plasma virus loads confirms the relatively low susceptibility of the study animals (Figure 17). Only 2 of the 6 control animals maintain a high virus load,  
20 while the virus disappears from the plasma in the other animals. Thus, a vaccine effect is difficult to demonstrate for the virus load parameter.

## Conclusions

25 Analysis of CD4-positive cells indicates that the vaccine gp120/NefTat + adjuvant (in the presence of SIV Nef) prevents the drop of CD4-positive cells in most vaccinated animals. This is a confirmation of the result obtained in the first SHIV study. Due to the lack of susceptibility of the study animals, the virus load parameter could not be used to demonstrate a vaccine effect. Taken together, the combination of gp120 and  
30 Tat and Nef HIV antigens provides protection against the pathologic consequences of HIV infection, as evidenced in a SHIV model.

The Tat alone antigens in combination with gp120 also provide some protection from the decline of CD4-positive cells. The effect is less pronounced than with the  
35 gp120/NefTat/SIV Nef antigen combination, but it demonstrates that gp120 and Tat are able to mediate some protective efficacy against SHIV-induced disease manifestations.

5

The second SHIV challenge study was performed with rhesus monkeys from a source completely unrelated to the source of animals from the first study. Both parameters, % of CD4-positive cells and plasma virus load, suggest that the animals in the second study were less susceptible to SHIV-induced disease, and that there was considerably greater variability among the animals. Nonetheless, a beneficial effect on the maintenance of CD4-positive cells of the gp120/NefTat/SIV Nef vaccine was seen with the experimental vaccine containing gp120/NefTat and SIV Nef. This indicates that the vaccine effect was not only repeated in a separate study, but furthermore demonstrated in an unrelated monkey population.

15

#### **Example 15: Generation of PMID vectors for gp120 and Nef/Tat**

Expression vectors were constructed as described below. Endotoxin free plasmid preps were prepared and used to transfect subconfluent 293T cell monolayers in 24 well tissue culture plates with 1µg of DNA using Lipofectamine 2000. Samples were harvested 24 hours post transfection and examined by Western Blot to assess expression levels. (Fig 18 and 19)

Codon optimisation of gp120 resulted in a substantial increase of REV independent expression.

25

#### ***Generation of plasmids***

See also Figures 20 – 23

30

#### **gp120:**

Both wild type and optimised gp120 were compared.

#### ***Wild Type: (pgp120w)***

35 PCR cloned from pRIT 13968 (see Example 11) using primers:

gp120w-5'      GAATTCGCGCCGCAATGAAAGTGAAGGAGACCAG

(Seq ID No: 32)

5 g120w-3' GAATTCGGATCCTTATCTCTGCACCACTCTTC  
(Seq ID No: 33)

The gene was cloned into vector p7313-ie as a NotI-BamHI fragment and sequenced.  
A single base change (conservative) was found relative to the reference sequence, but  
10 this change was also found on sequencing pRIT 13968 (T1170C in gp120).

*Codon Optimised (pgp120c)*

The gene sequence was based on the gp120 sequence from pRIT 13968. This has a  
RSCU value of 0.297. Optimisation was performed using SynGene 2d, resulting in a  
15 RSCU value of 0.749. The sequence was split into 40 overlapping oligonucleotide,  
PCR assembled and recovered using the end primers. The gene was cloned into vector  
p7313-ie as a NotI-BamHI fragment and sequenced. Restriction fragments from three  
initial clones were combined to generate a single correct clone.

20 **Nef/Tat (pNTm and ptrNTm)**

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244. The  
plasmid pRIT 15244 is identical to pRIT 14913 previously described except that the  
His tail has been deleted. The Tat in this plasmid contains three mutations as  
previously described. The fusion contains full length Nef which has an immune  
25 modulatory function (Collins and Baltimore (1999)) that may be abrogated by N-  
terminal truncation. Therefore constructs were generated for both full length  
Nef/mutant Tat(pNTm) and truncated Nef/mutant Tat(ptrNTm), in which the first 65  
amino acids of Nef were removed. These sequences were PCR amplified from  
pRIT15244 using primers:

30

5'Nef: GAATTCGCGGCCGCCATGGGTGGCAAGTGGTCAAAAAG  
5'trNef: GAATTCGCGGCCGCCATGGTGGGTTTTCCAGTCACACC  
3'Tat: GAATTCGGATCCTTATTCCTTCGGGCCTGTCGGG  
(Seq ID Nos: 34, 35 and 36 respectively)

35 The genes were cloned into vector p7313-ie as NotI-BamHI fragments and sequenced.

**Dual expression vectors: (pRIX1 and pRIX2)**

5

The Nef/Tat and trNef/Tat expression cassettes were excised as ClaI-XmnI restriction fragments, and ligated into the ClaI and blunted Sse8387 I sites of the vector containing the codon optimised gp120 to provide single plasmids for expression of both proteins (pRIX1 and pRIX2 respectively).

10

#### Composition of plasmid p7313-ie

The plasmid was constructed by replacing the beta-lactamase gene containing Eam1105I - Pst1 fragment of pUC19 (available from Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks, HP7 9NA) with an EcoRI fragment of pUC4K (Amersham-Pharmacia) containing the Kanamycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus IE1 promoter /enhancer, Intron A, was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and inserted into the Sal1 site of pUC19 as a XhoI -Sal1 fragment, incorporating the bovine growth hormone polyadenylation signal. Deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector (WO00/23592 - Powderject Vaccines Inc.). HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA, 78, 2606-10, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with *Bgl* II, Klenow polymerase treatment, digestion with *BstX* I, digestion with *Nco* I, treatment with mung bean nuclease to remove overhang and further digestion with *BstX* I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids) and the X gene start codon. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and

35

- 5 coding sequences of the S antigen were excised and replaced with an oligonucleotide linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

Hind---NotI-- EcoRV- -NdeI- -BamHI

- 10 AGCTTGCGGCCGCTAGCGATATCGGTACCATATGTCGACGGATCC....  
 ....ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGCCTAGGCCGG  
 -NheI- -KpnI- -SalI- ΔNotI  
 (Seq ID No: 37)

- 15 This polylinker was further extended by insertion of an additional oligonucleotide linker between the KpnI and SalI sites:

- AspI- -MunI- NaeI- NdeI-- BglII-  
 GTACCGGTCAATTGGCGCCGGCGCGCCATATGACGTCAGATCTG----  
 20 ----GCCAGTTAACCGCGGCCGCGCGGTATACTGCAGTCTAGACAGCT  
 --AgeI- -NarI-- AatII- SalI  
 (Seq ID No: 38)

- The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from  
 25 David Hodgeson (Warwick University) and amplified by PCR using primers to place EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the amplified cer was verified against the Genbank entry M11411.

- The HBV 3'UTR sequence between the promoter and polyadenylation signal was  
 30 removed by PCR amplification of the polyadenylation signal using primers:

sense: CCATGGATCCGATCTTTTCCCTCTGCC (Seq ID No: 39)

antisense: GTTAGGGTGAAAAGCTTCCGAGTGAGAGACAC (Seq ID No: 40)

The resulting product was cut with BamHI and XmnI and used to replace the corresponding fragment containing both the polyadenylation signal and the 3'UTR.

- 35 The Intron A sequence was removed from the plasmid by PCR amplification of the CMV promoter/enhancer using primers:

sense: GCTAGCCTGCAGGCTGACCGCCCAACGAC (Seq ID No: 41)



- 5 antisense: GTTCTCCATCGCGGCCGCACTCTTGGCACGGGG (Seq ID No: 42)  
The resulting product was cut with Sse8387 I and NotI, and inserted back into the  
Sse8387 I and NotI sites of the parental vector.

## 10 **Example 16: IMMUNOGENICITY STUDIES**

- A mouse immunogenicity study may be performed to test the immunogenicity of  
gp120 and NefTat constructs delivered by particle mediated immunotherapeutic  
delivery (PMID) to show that the construct can generate an immune response *in vivo*.  
15 The DNA constructs may be used in combination with protein to determine whether  
there is an advantage of the combined approach compared to immunisation with the  
individual components. The aim is to detect and quantify cellular and humoral  
immune responses in mice following priming with protein and boosting with DNA  
(PMID) or priming with DNA (PMID) and boosting with protein.  
20 The DNA is precipitated onto gold microparticles, which are used to coat the inner  
walls of a tefzel cartridge. PMID cartridges are prepared using a DNA loading rate of  
2, which gives approximately 0.5 µg DNA per cartridge.  
25 Mice are immunised by PMID on the abdomen by providing two shots containing  
gp120 or NefTat. Following a PMID boost spleens are removed and the spleen cells  
are used to determine immunogenicity to individual constructs. Responses are  
evaluated using the IFN-γ ELISpot assay. In this assay peptides from gp120 and Nef  
are used to stimulate the spleen cells to secrete IFNγ which is captured by an antibody  
30 based detection system. The measurement of humoral responses to gp120 and Nef are  
measured by ELISA.

### **gp120/NefTat Prime Boost Studies**

- 35 The aim of the study is to detect and quantify cellular and humoral immune responses  
in mice following priming with protein and boosting with DNA (PMID) or priming  
with DNA (PMID) and boosting with protein.

5

Cartridge Preparation

Gene gun cartridges are suitably prepared using a DNA loading rate (DLR) of 2, which will give approximately 0.5 µg DNA per cartridge.

10

In vivo Immunogenicity

15

An experiment (Experiment I) may suitably be carried out as set out in the table below. The readouts for this experiment are carried out by IFN-γ ELISpot to Balb/c peptides from gp120 and Nef to measure the cellular responses and detection of antibodies to gp120 and Nef by ELISA for the humoral response.

**Experiment I**

Group	Mouse strain	Prime	Boost
A	Balb/c Female	PMID: gp120, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg
B	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	PMID: NefTat, 2 x 0.5 µg
C	Balb/c Female	PMID: Empty vector, 2 x 0.5 µg	PMID: Empty vector, 2 x 0.5 µg
D	Balb/c Female	Protein: gp120	Protein: gp120
E	Balb/c Female	Protein: NefTat	Protein: NefTat
H	Balb/c Female	Protein: Irrelevant protein	Protein: Irrelevant protein

n = 3

20

A further experiment (Experiment II) may be carried out as follows:

**Experiment II**

Group	Mouse strain	Prime	Boost
A	Balb/c Female	PMID: gp120, 2 x 0.5 µg	Protein: gp120
B	Balb/c	PMID: gp120, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg

	Female		
C	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	Protein: NefTat
D	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	PMID: NefTat, 2 x 0.5 µg
E	Balb/c Female	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg	Protein: gp120 + NefTat
F	Balb/c Female	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg
G	Balb/c Female	PMID: Empty vector, 2 x 0.5 µg	Protein: Irrelevant protein
H	Balb/c Female	Protein: gp120	PMID: gp120, 2 x 0.5 µg
I	Balb/c Female	Protein: gp120	Protein: gp120
J	Balb/c Female	Protein: NefTat	PMID: NefTat, 2 x 0.5 µg
K	Balb/c Female	Protein: NefTat	Protein: NefTat
L	Balb/c Female	Protein: gp120 + NefTat	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg
M	Balb/c Female	Protein: gp120 + NefTat	Protein: gp120 + NefTat
N	Balb/c Female	Protein: Irrelevant protein	PMID: Empty vector, 2 x 0.5 µg

5 n = 3

### Sample protocol

10 Female Balb/c mice are used. All mice are pre-bled (tail vein) before the start of the experiment.

Groups of 3 mice/group are culled after the primary immunisation and spleens are collected for enumeration of IFN-γ -producing CD8 cells by ELISpot assay using gp120 and Nef K<sup>d</sup>-restricted peptides.

15 Groups of 3 mice/group are culled at a minimum of two time points after the secondary immunisation and cardiac blood samples are collected. These are analysed for antibodies to gp120 and Nef using ELISA assays. Spleens are also be collected for enumeration of IFN-γ -producing CD8 cells by ELISpot assay using gp120 and Nef K<sup>d</sup>-restricted peptides.

5

## **Immunological Assays**

### ELISpot assays

Cellular immune responses are detected using Interferon- $\gamma$  ELISpot assays.

- 10 Splenocytes will be isolated from 3 mice per group at each selected time point (one post primary and a minimum of two post-boost), and incubated overnight with peptides to known CD8 epitopes (restricted to a K<sup>d</sup> (Balb/c) background) in plates coated with  $\alpha$ -Interferon- $\gamma$ . The splenocytes are lysed and plates developed using a secondary  $\alpha$ -Interferon- $\gamma$  antibody and biotin-streptavidin amplification system.

15 ELISA assays

Humoral responses are detected using standard antibody ELISA assays. 96-well flat-bottomed microtitre plates will be coated with protein and blocked. Serial dilutions of serum collected prior to immunisation and after boost are collected and incubated in the plates. The plates are developed after incubation with an anti-mouse antibody.

- 20 Responses to gp120, Nef and Tat are analysed.

## **Example 17: IMMUNOGENICITY STUDIES**

25 **Protocol**

Cartridges were prepared for particle mediated delivery using a gene gun using standard methods. A DNA loading rate of 2 which will give approximately 0.5  $\mu$ g DNA/cartridge was used.

- 30 F1 (C3H x Balb/c) mice were given a primary immunisation with either gp120 protein and NefTat in adjuvant (administered via the intramuscular route) or with gp120 DNA, codon optimised and cloned into vector p7313-ie as described in Example 15, and a vector expressing a Nef fusion protein (using particle mediated delivery wherein the DNA is coated onto gold beads). The mice were boosted 23 days later either with
- 35 protein in adjuvant (administered via the intramuscular route) or with DNA (using particle mediated delivery wherein the DNA is coated onto gold beads). Mice were culled 5 days later and spleens were collected. The splenocytes were harvested by

5 teasing out, erythrocytes were lysed and splenocytes were washed and counted. Specialised ELISpot plates (coated with interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37°C/5% CO<sub>2</sub> in the presence of medium control gp120 E7 peptide or various nef peptides. The splenocytes were lysed and the plate developed using  
10 standard procedures to demonstrate the number of interferon-gamma secreting cells present.

### Conclusion

Although the ELISpot assay was carried out using a sub-optimal concentration of  
15 gp120 E7 peptide, the results indicated that the most effective schedule was to prime mice with protein in adjuvant (administered i.m.) and then boost with DNA (administered by particle mediated delivery). See Figures 24. Similar results were obtained for nef, with the two peptides Nef 19 and 20 only being recognised by mice that had been primed with protein and boosted with DNA. The sequences for these  
20 two peptides are Nef 29: HIV-1 Bru (171-190) GMDDPEREVLEWRFD SRLAF (Seq ID No: 43) and Nef 20: HIV-1 Bru (181-200) EWRFD SRLAFHHVARELHPE (Seq ID No: 44). See Figure 25.

### Example 18: IMMUNOGENICITY STUDIES

25

#### Protocol

For PMID immunisations (DNA) cartridges were prepared using standard methods. A DNA loading rate of 2, which will give approximately 0.5 µg DNA/cartridge was used and each immunisation consisted of two shots. Protein was formulated in adjuvant  
30 comprising squalene/tocopherol/Tween 80/3D-MPL/QS21 just before use. Balb/c mice were given a primary immunisation of either gp120 protein in adjuvant (administered via the intramuscular route) or with gp120 codon optimised DNA prepared as described in Example 15 (using PMID). The mice were boosted 21 days later with either protein in adjuvant (administered via the intramuscular route) or with  
35 DNA (using PMID). Mice were culled 7 days later and spleens were collected. The splenocytes were harvested by teasing out the spleen cells and erythrocytes were lysed. The splenocytes were washed and counted. Specialised ELISpot plates (coated with

5 interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37°C/5% CO<sub>2</sub> in the presence of pools of gp120 15-mer peptides. The splenocytes were lysed and the plate developed using standard procedures to demonstrate the number of interferon-gamma secreting cells present. Results are shown in Figure 26.

10

### **Conclusion**

Three pools of gp120 15-mer peptides were recognised by mice that had been primed with protein and boosted with DNA. Responses to these three pools of gp120 15-mers were not detected in animals that had been primed with DNA and boosted with

15 protein or immunised twice with either protein or DNA.

5

## CLAIMS

1. Use of a) an HIV Tat protein or polynucleotide; or  
b) an HIV Nef protein or polynucleotide; or  
c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or  
10 polynucleotide;  
and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable  
for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans  
against HIV, wherein the protein or polynucleotide is delivered via a bombardment  
approach.
- 15 2. Use according to claim 1 wherein the bombardment approach comprises  
propelling particles into a target tissue of interest, typically the skin.
3. Use according to claim 2 wherein the particles are gold beads onto which the  
protein or polynucleotide has been coated.
4. Use according to claim 2 or claim 3 in which the particles are accelerated to  
20 high speed by a helium gas jet.
5. Use according to any one of claims 2 to 4 wherein the gold beads are 0.4-4.0  
µm in diameter
6. Use according to claim 5 wherein the gold beads are 0-6 to 2.0 µm in diameter.
7. Use according to any preceding claim wherein the polynucleotide encoding the  
25 nef, tat or gp120 is codon-optimised DNA.
8. Use according to any preceding claim in which the polynucleotide encoding  
the nef, tat and gp120 is present on a single vector.
9. Use according to claim 8 in which the vector comprises the nef, tat and gp 120  
polynucleotides inserted 3' to an enhanced HCMV IE1 promoter.
- 30 10. Use according to claim 9 in which the vector is p7313.
11. Use according to any preceding claim in which additional regulatory or  
structural proteins of HIV such as Rev, Vif, Vpu, and Vpr or proteins derived from the  
HIV *gag* or *pol* genes (and/or polynucleotides encoding such regulatory or structural  
proteins) are included in the vaccine formulation.
- 35 12. A recombinant DNA molecule comprising a Nef and/or Tat and/or gp 120  
gene in a vector in which the gene of interest is inserted 3' to an enhanced HCMV IE1  
promoter.

- 5 13. A recombinant DNA molecule according to claim 12 in which the vector is p7313.
14. A recombinant DNA molecule according to claim 12 or claim 13 in which at least one of the genes of interest is codon-optimised.
15. A recombinant DNA molecule in which the gp 120 DNA is codon-optimised.
- 10 16. Use of a recombinant DNA molecule according to any one of claims 12 to 15 in the manufacture of an HIV vaccine for the prophylactic or therapeutic immunisation of humans.
17. A plurality of particles, preferably gold particles, coated with recombinant DNA comprising a Nef and/or Tat and/or gp 120 gene in a vector.
- 15 18. Particles according to claim 17 coated with DNA comprising a Nef, a Tat and a gp 120 gene in a single vector, preferably wherein the Nef and Tat gene are in the form of a NefTat fusion.
19. Particles according to claim 17 or 18 wherein the DNA encoding at least one of the Nef, Tat or gp120 is codon optimised for expression in human cells.
- 20 20. Particles according to any one of claims 17 to 19 wherein one or more of the genes of interest is inserted 3' to an enhanced HCMV IE1 promoter.
21. Particles according to any one of claims 17 to 20 in which the vector is P7313.
22. A kit comprising at least two different vaccine compositions including a) a composition comprising a plurality of particles coated with DNA encoding gp120 and
- 25 nef and/or tat or nefat as described herein and b) a composition comprising gp120 and nef and/or tat or nefat DNA or proteins as described herein wherein the DNA or proteins in b) are not coated onto particles.



**FIGURE 1**

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

Pichia-expressed constructs (plain constructs)

⇒ Nef - HIS

DNA sequence (Seq. ID. No. 8)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA  
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA  
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG  
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA  
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTTAAAAGAAAAGGGG  
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC  
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC  
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG  
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT  
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA  
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC  
CACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 9)

MGGKWSKSSVVGWPTVRERMRRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW  
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWI  
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLHPVSLH  
GMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ Tat - HIS

DNA sequence (Seq. ID. No. 10)

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAA  
ACTGCTTGTTACCAATTGCTATTGTAAAAAGTGTGCTTTCATTGCCAAGTTTGTTC  
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA  
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAA

TCCCGAGGGGACCCGACAGGCCCGAAGGAACTAGTGGCCACCATCACCATCACCAT  
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNICYCKKCCFHCQVCFITKALGISYGRKKRRQRRR  
PPQGSQTHQVLSKQPTSQSRGDPTGPKETSGHHHHHH.

⇒ Nef - Tat - HIS

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA  
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA  
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG  
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA  
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG  
GGACTGGAAGGGCTAATTCCTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC  
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC  
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG  
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT  
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA  
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG  
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAACTGCT  
TGTACCAATTGCTATTGTAAAAGTGTTGCTTTTCATTGCCAAGTTTGTTCATAACA  
AAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCT  
CAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGA  
GGGGACCCGACAGGCCCGAAGGAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 13)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW  
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWI  
YHTQGYFPDWQNYTPGPGVRYPLTFGWICYKLVPEPDKVEEANKGENTSLHPVSLH  
GMDDPEREVLEWRFSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA  
CTNICYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVLSKQPTSQSR  
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ LipoD-Nef-HIS

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.  
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

\*

ATGGATCCAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT  
 AGCAGCCATTCATCAAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT  
 GCTCACCGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT  
 GCTTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT  
 CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA  
 TTCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA  
 GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA  
 AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA  
 GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA  
 AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG  
 GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG  
 GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT  
 CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC  
 TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT  
 GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA  
 GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG  
 AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCTAGCATTTTCATCACGTGGCCCGA  
 GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCACCATCACCAT  
 TAA

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKD  
 GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW  
 SKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE  
 EEEVGFVPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWIYHTQG  
 YFPDQWNYTPGPGVRYPLTFGWCYKLVPVEPKVVEANKGENTSLHHPVSLHGMDDP  
 EREVLEWRFD SRLAFHHVARELHPEYFKNCTSGHHHHHHH.

⇒ LipoD-Nef-Tat-HIS

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.  
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

\*

ATGGATCCAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT  
AGCAGCCATTCATCAAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT  
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACTAGTAATCTAAAGCACTT  
GCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT  
CGTTTAGTGGTTATTCACGATCACTTTTATAGATGGCTTGACTGATGTTGCGAAAAA  
TTCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA  
GAAATTCAAAGTTTAGAAATGACAGAAAACCTTGAAACCATGGGTGGCAAGTGGTCA  
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA  
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA  
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG  
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG  
GCAGCTGTAGATCTTAGCCACTTTTTTAAAGAAAAGGGGGGACTGGAAGGGCTAATT  
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC  
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCACTGACCTTT  
GGATGGTGTCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA  
GGAGAGAACACCAGCTTGTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG  
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA  
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA  
GAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACCTGCTTGTACCAATTGCTATTGT  
AAAAAGTGTTGCTTTCATTGCCAAGTTTGTTCATAACAAAAGCCTTAGGCATCTCC  
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT  
CAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCG  
AAGGAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKD  
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRIYVIDFTLKEIQSLEMTENFETMGKW  
SKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGATTSSNTAATNAACAWLEAQE  
EEEVGFVPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWIYHTQG  
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP  
EREVLEWRFD SRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY  
CKKCCFHCQVCFITKALGISYGRKKRRQRRRPQGSQTHQVSLSKQPTSQSRGDPTG  
PKETSGHHHHH.

⇒ ProtD-Nef-HIS

DNA sequence (Seq. ID. No. 18)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA  
 ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATAACGTTAGAATCT  
 AAAGCACTTGCGTTTGACAAACAGGCTGATTATTTAGAGCAAGATTTAGGAATGACT  
 AAGGATGGTTCGTTTAGTGGTTATTACGATCACTTTTTAGATGGCTTGACTGATGTT  
 GCGAAAAAATCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT  
 ACCTTAAAGAAATTCAAAGTTTAGAAATGACAGAAAACCTTGAAACCATGGGTGGC  
 AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA  
 GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA  
 GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTCCTGGCTAGAAGCA  
 CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG  
 ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAGAAAAGGGGGGACTGGAA  
 GGGCTAATTCCTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA  
 CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCA  
 CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG  
 GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT  
 GACCCTGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTATCAC  
 GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC  
 CATCACCATTAA

Protein sequence (Seq. ID. No. 19)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIHAHRGASGYLPEHTLESKALAFQAQADYL  
 EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK  
 EIQSLEMTENFETMGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRD  
 LKHXGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH  
 FLKEKGGLEGLIHSQRRQDILDWYHTQGYFPDWQNYTPGPGVRYPLTFGW  
 CYKLVPVEPDKVEEANKGENTSLHPVSLHGMDDPEREVLEWRFDRLAFH  
 HVARELHPEYFKNCTSGHHHHHH.

⇒ ProtD-Nef-Tat-HIS

DNA sequence (Seq. ID. No. 20)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA  
 ATCATTTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACTGTTAGAATCT  
 AAAGCACTTGCCTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT  
 AAGGATGGTCGTTTAGTGTTATTCACGATCACTTTTATAGATGGCTTGACTGATGTT  
 GCGAAAAAATCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT  
 ACCTTAAAGAAATTCAAAGTTTAGAAATGACAGAAAACCTTGAAACCATGGGTGGC  
 AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA  
 GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA  
 GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA  
 CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG  
 ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTTAAAGAAAAGGGGGGACTGGAA  
 GGGCTAATTCCTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA  
 CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCA  
 CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG  
 GCCAATAAAGGAGAGAAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT  
 GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCTAGCATTTCATCAC  
 GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT  
 CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACCTGCTTGTACCAAT  
 TGCTATTGTAAAAGTGTTGCTTTTATTGCCAAGTTTGTTCATAACAAAAGCCTTA  
 GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGT  
 CAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCG  
 ACAGGCCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 21)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMT  
 KDGRLLVVIHDHFLDGLTDVAKKFPHRHRKDGRIYVIDFTLKEIQSLEMTENFETMGG  
 KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEA  
 QEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWIYHT  
 QGYFPDWQNYTPGPGVRYPLTFGWICYKLVPEPDKVEEANKGENTSLLHPVSLHGMD  
 DPEREVLEWRFDRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTN  
 CYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDP  
 TGPKETSGHHHHHH.

⇒ Tat-MUTANT-HIS

DNA sequence (Seq. ID. No. 22)

```

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC 40
CAGGAAGTCAGCCTAAACTGCTTGTACCAATTGCTATTG 80
TAAAAAGTGTGCTTTCATTGCCAAGTTTGTTCATAACA 120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC 160
AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGT 200
TTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG 240
CCGACAGGCCCGAAGGAACTAGTGGCCACCATCACCATC 280
ACCATTAA 288

```

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

```

MEPVDPRLEPWKHPGSQPKTACTNICYCKKCCFHCQVCFIT 40
AALGISYGRKKRRRQRRRPPQGSQTHQVSLSKQPTSQSKGE 80
PTGPKETSGHHHHHHH. 95

```

⇒Nef-Tat-Mutant-HIS

DNA sequence(Seq. ID. No. 24)

```

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGC 40
CTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC 80
AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACAT 120
GGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTG 160
CTTGTGCCTGGCTAGAAGCACAAAGAGGAGGAGGAGGTGGG 200
TTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACT 240
TACAAGGCAGCTGTAGATCTTAGCCACTTTTTTAAAAGAAA 280
AGGGGGGACTGGAAGGGCTAATTCACCTCCAACGAAGACA 320
AGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC 360
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCA 400
GATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACC 440
AGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAG 480
AACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGG 520
ATGACCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAG 560
CCGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCG 600
GAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTA 640
GACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAC 680
TGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT 720
TGCCAAGTTTGTTCATAACAGCTGCCTTAGGCATCTCCT 760
ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCA 800
AGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCC 840
ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAAA 880
CTAGTGGCCACCATCACCATCACCATTAA 909

```

Protein sequence (Seq. ID. No. 25)

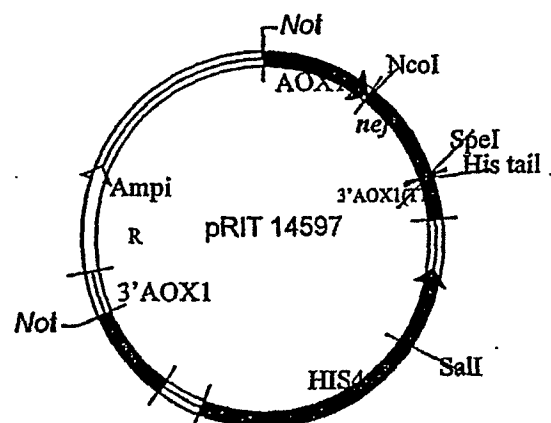
Mutated amino-acids in Tat sequence are in bold.

MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKH 40  
GAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMT 80  
YKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGY 120  
FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE 160  
NTSLLHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHP 200  
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH 240  
CQVCFITAALGISYGRKKRRQRRPPQGSQTHQVSLSKQP 280  
TSQSKGEPTGPKETSGHHHHHH. 302



Figure 2

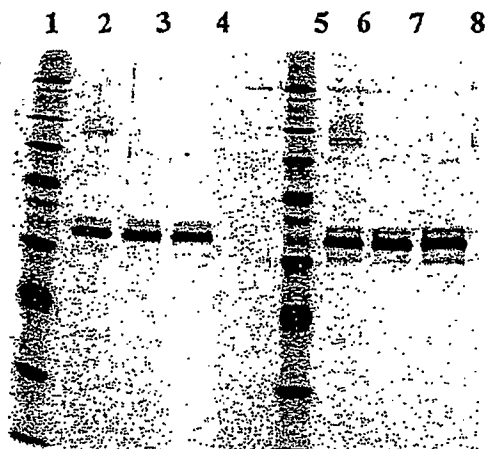
## Map of pRIT14597 integrative vector



MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

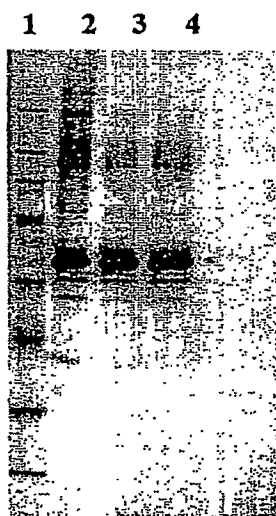
<i>Asu</i> II	<i>Nco</i> I	<i>Spe</i> I	<i>Eco</i> RI
TTCGAA	<u>ACC.ATGGCCGCGG</u>	<u>ACTAGTGGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA</u>	<u>CGGAATTC</u>
		Thr . Ser . Gly . His . His . His . His . His . His	

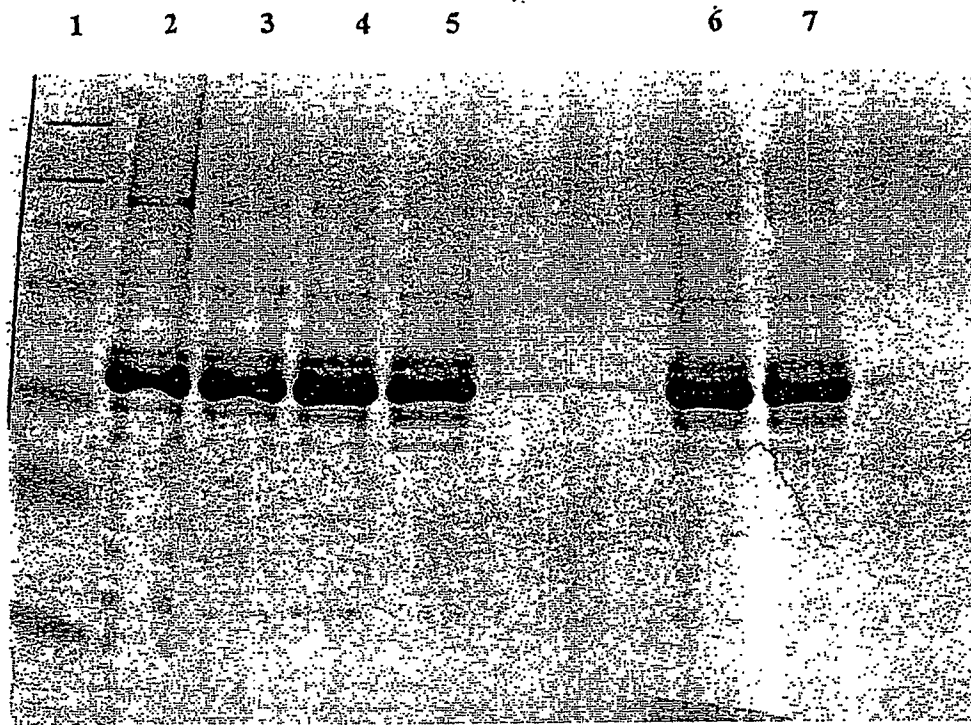
The amino acid sequence of Figure 3 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No. 26.

**Figure 3 : SDS-PAGE: Nef-Tat-his fusion protein****Daiichi Silver Staining**

- 1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
 2: TNH/23 SP eluate (250 ng)  
 3: TNH/23 Purified bulk (250 ng)  
 4: TNH/22 Purified bulk (250 ng)

- 5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
 6: TNH/23 SP eluate (400 ng)  
 7: TNH/23 Purified bulk (400 ng)  
 8: TNH/22 Purified bulk (400 ng)

**Blot  $\alpha$  Nef-Tat (LAS 97340)****Blot Tat2**

**Figure 4 : SDS-PAGE: Nef-Tat-his fusion protein**

**Coomassie blue G250**

**1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)**

**2: TNH/23 SP eluate (4 µg)**

**3: TNH/23 Superdex200 eluate (4 µg)**

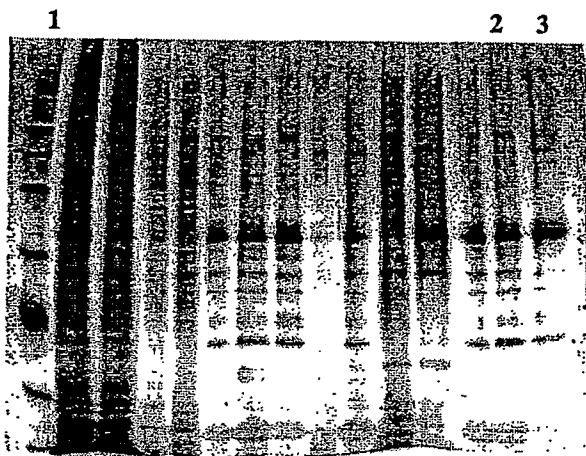
**4: TNH/23 Purified bulk (4 µg)**

**5: TNH/22 Purified bulk (4 µg)**

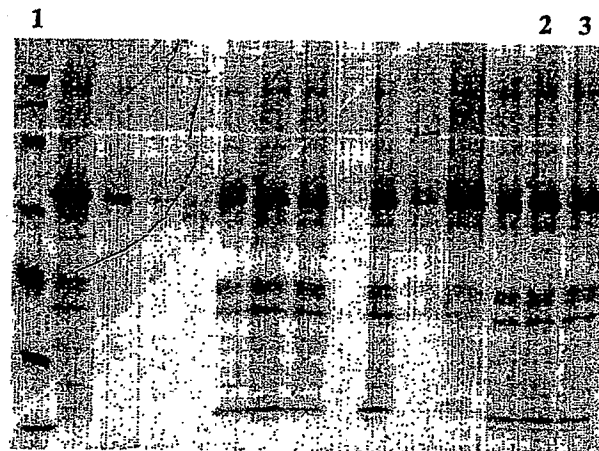
**6: TNH/23 Purified bulk (4 µg) / non reducing conditions**

**7: TNH/22 Purified bulk (4 µg) / non reducing conditions**

**Figure 6: SDS-PAGE ANALYSIS – reducing conditions**  
**(14% polyacrylamide precasted gels - Novex) See example 5**



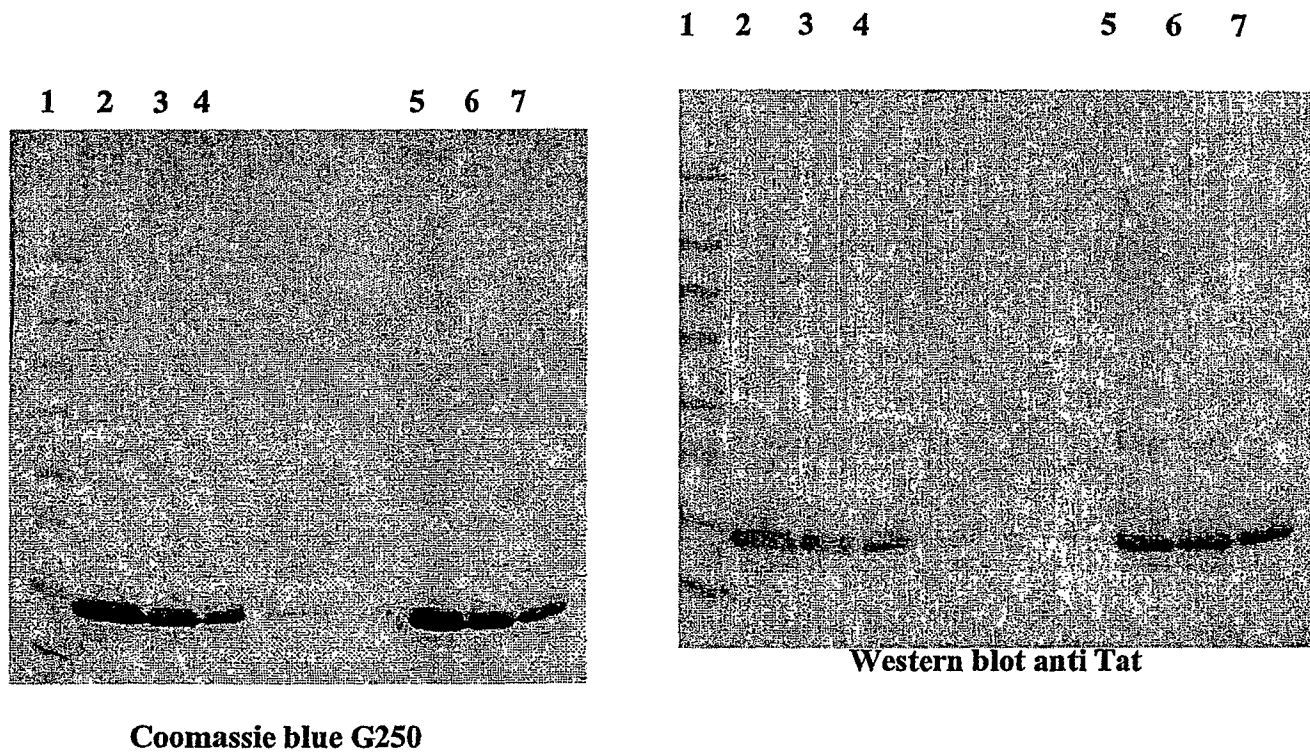
**Silver staining**



**Western blot  $\alpha$  Tat**

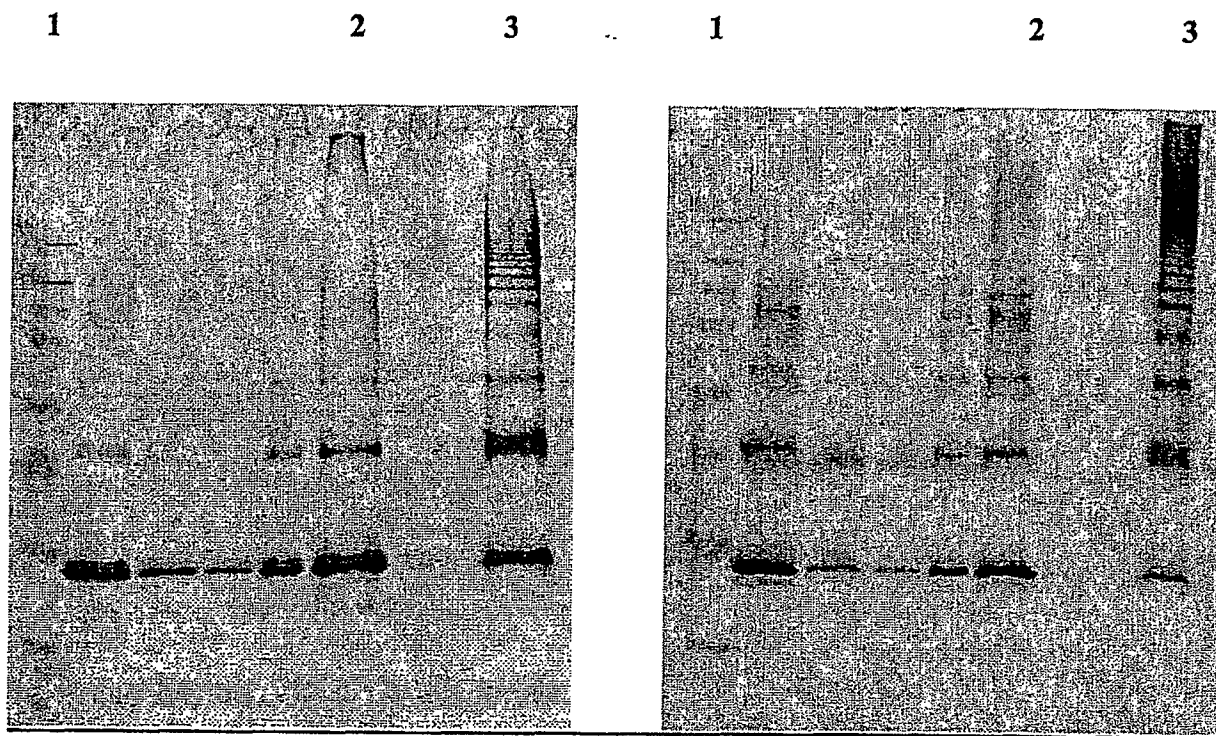
- 1: MW (175/83/62/47,5/32,5/25/16,5/6,5 kDa)**  
**2: Purified bulk**  
**3: Purified bulk**

**Figure 7 (relating to Example 6): SDS-PAGE ANALYSIS:**  
 (4-20% polyacrylamide precasted gels - Novex)



- 1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
 2: Purified bulk (reducing conditions)  
 3: Purified bulk (reducing conditions)  
 4: Purified bulk (reducing conditions)  
 5: Purified bulk (non reducing conditions)  
 6: Purified bulk (non reducing conditions)  
 7: Purified bulk (non reducing conditions)

**Figure 8 (relating to Example 7): SDS-PAGE ANALYSIS:**  
(4-20% polyacrylamide precasted gels - Novex)



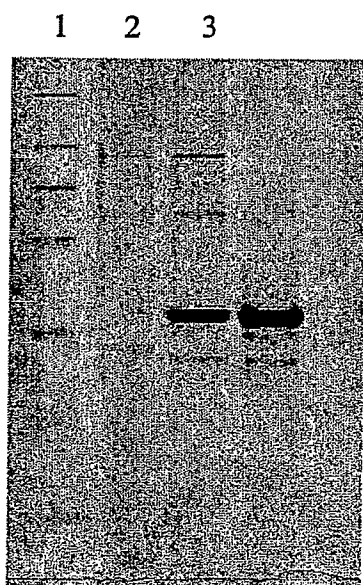
**Coomassie blue G250**

**Western blot anti Tat**

- 1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
2: Purified bulk (reducing conditions)  
3: Purified bulk (non reducing conditions)

**FIGURE 9: SDS-PAGE ANALYSIS - REDUCING CONDITIONS**

(14% polyacrylamide precasted gels - Novex) see Example 8



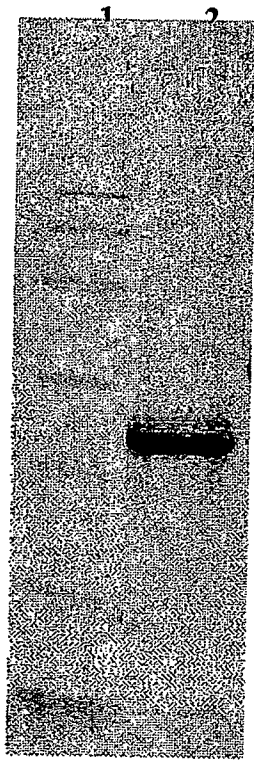
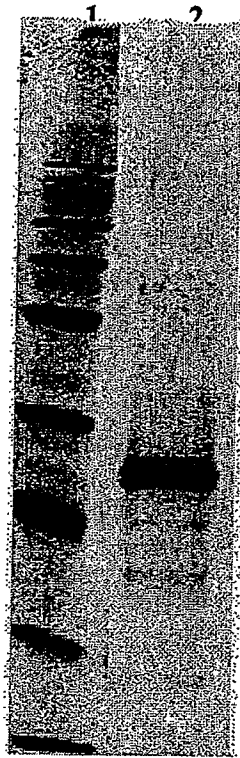
**Coomassie blue R250**



**Silver staining**

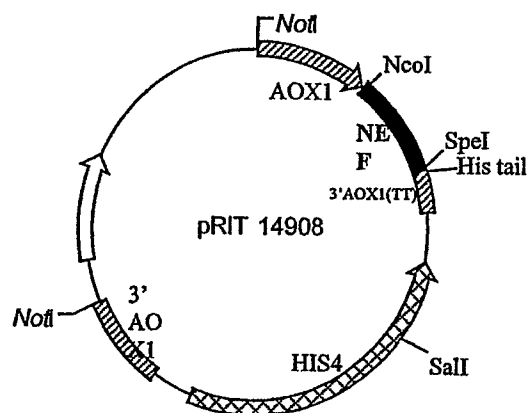
**Figure 10: SDS-PAGE ANALYSIS – reducing conditions**

(14% polyacrylamide precasted gels - Novex) See Example 9

**Coomassie blue G250****Silver staining****W. Blot  $\alpha$  Nef**

**1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)**  
**2: Purified bulk**



**Figure 11****Map of pRIT14908 integrative vector**

MCS POLYLINKER : NEF gene inserted between NcoI and SpeI sites.

<i>Asu</i> II	<i>Nco</i> I	<i>Spe</i> I	<i>Eco</i> RI
TTCGAA.A	<u>CC.ATG</u> GCCGCGG	ACTAGT	.GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGC
			<u>GAATTC</u>
			Thr .Ser . Gly. His . His . His . His . His . His .

Figure 12

## Sequences of Pichia-expressed SIV-NEF-His protein

DNA SEQUENCE:

```

atgggtggagctat t tccatgagggcgggtccaggccgtctggagatctgcg      50
acagagactcttgcggggcgcggtggggagacttatgggagactcttaggag      100
aggtggaagatgggatactcgcaatcccaggaggattagacaagggcttg      150
agctcactctcttgtgagggacagaaatacaatcagggacagtatatgaa      200
tactccatggagaaacccagctgaagagagagaaaaattagcatacagaa      250
aacaaaatatggatgatatagatgaggaagatgatgacttggtaggggta      300
tcagtgaggccaaaagttcccctaagaacaatgagttacaaattggcaat      350
agacatgtctcattttataaaaagaaaaggggggactggaagggaatttatt      400
acagtgaagaagacatagaatcttagacataacttagaaaaggaagaa      450
ggcatcataccagattggcaggattacacctcaggaccaggaattagata      500
cccaaagacatttggctggctatggaaattagtccctgtaaatgtatcag      550
atgaggcacaggaggatgaggagcattatttaatgcatccagctcaaact      600
tcccagtgggatgacccttggggagaggttctagcatggaagtttgatcc      650
aactctggcctacacttatgaggcatatgttagataccagaagagtttg      700
gaagcaagt caggcctgtcagaggaagaggttagaagaaggctaaccgca      750
agaggccttcttaacatggctgacaagaaggaaactcgcactagtggcca      800
ccatcaccatcaccattaa.                                         819

```

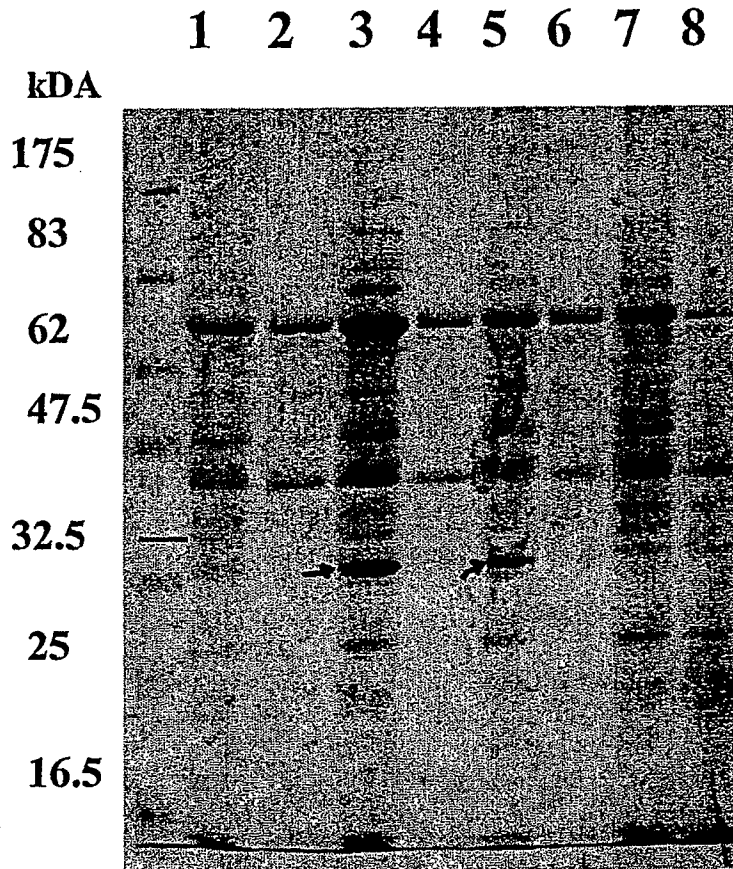
PROTEIN SEQUENCE:

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MGGAI SMRRSRPSGDLRQRLLRARGETYGRLLGEVEDGYSQSPGGLDKGL      50
SSLSC EGQKYNQGQYMNT PWRNPAEEREK LAYRKQNMDDIDEEDDDL VGV      100
SVRPKVPLRTMSYKLAIMSHFIKEKGGLEGIYYSARRHRILDIYLEKEE      150
GIIPDWQDYTS GPGIRYPKTFGWLWKLVPVNV SDEAQEDEEHYLMHPAQ T      200
SQWDDPWGEVLAWKFDPTLAYTYEAYVRYPEEFGSKSGLSEEEVRRRLTA      250
RGLLN MADKKETR TSGHHHHHH .                                  272

```

**Figure 13**  
**Coomassie Blue Stained SDS-PAGE of recombinant**  
***Pichia pastoris* SIV/NEF expressing strains**



lane 1: P- Y1752 strain  
lane 2: S- " "  
lane 3: P- Y1772 strain  
lane 4: S- " " "  
lane 7: P- GS115 strain ( negative control)  
lane 8: S- " "

Figure 14. Monkey study 1. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

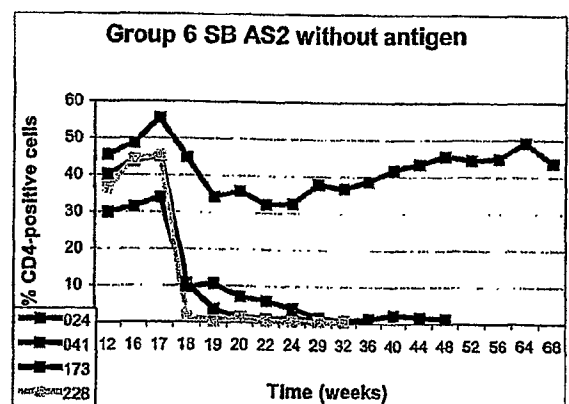
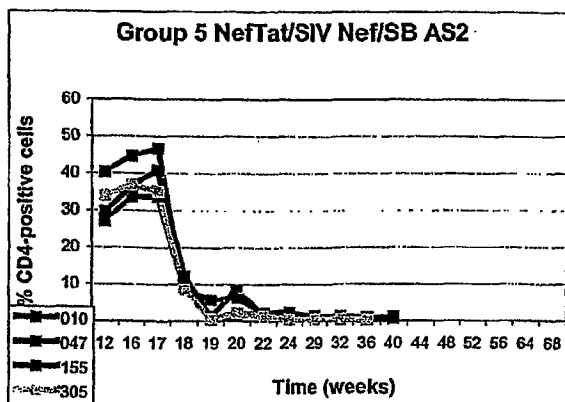
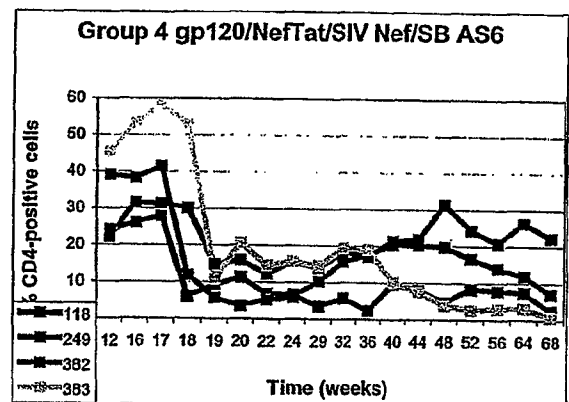
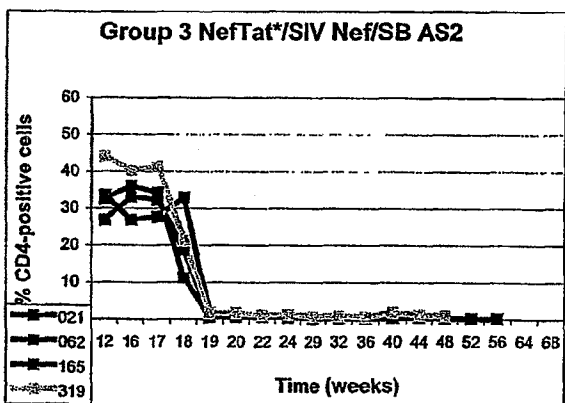
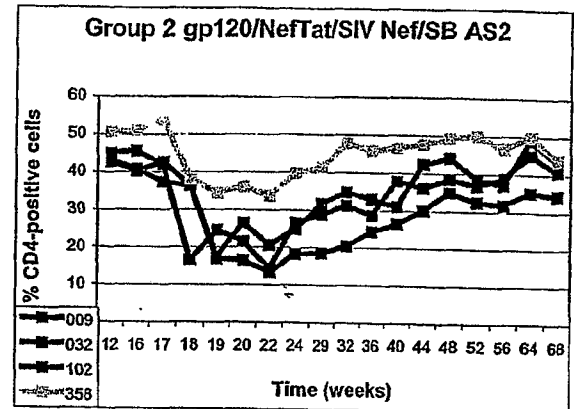
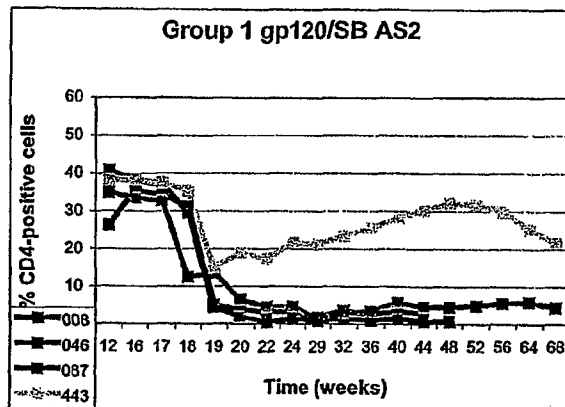


Figure 15. Monkey study 1. Analysis of SHIV plasma virus load after challenge with SHIV

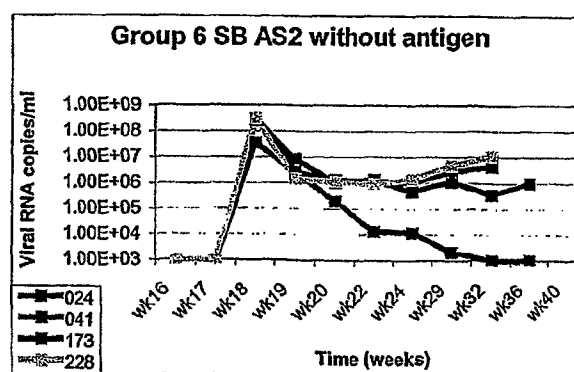
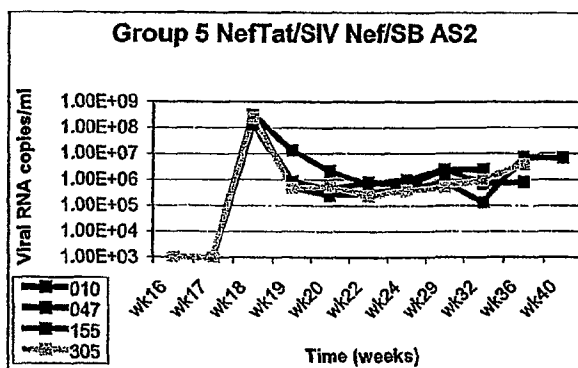
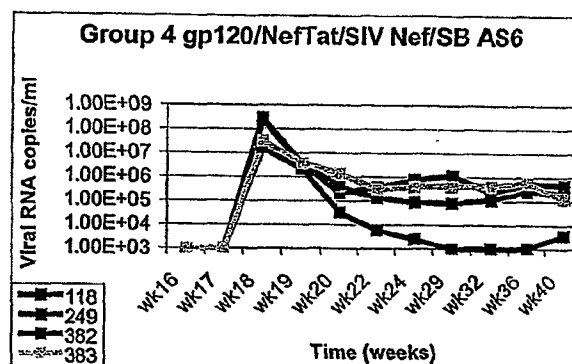
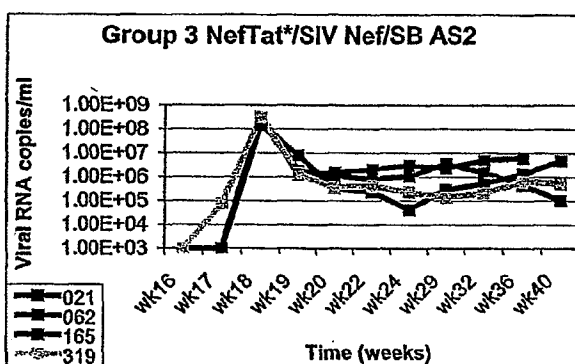
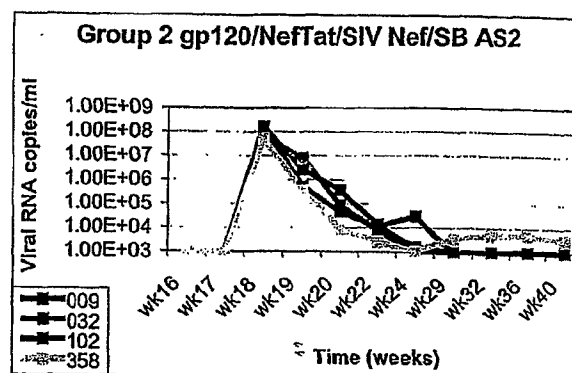
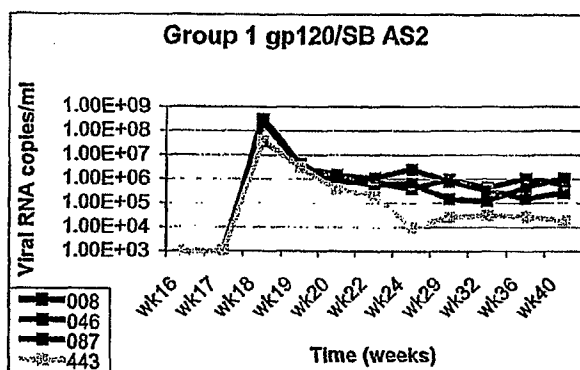


Figure 16. Monkey study 2. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

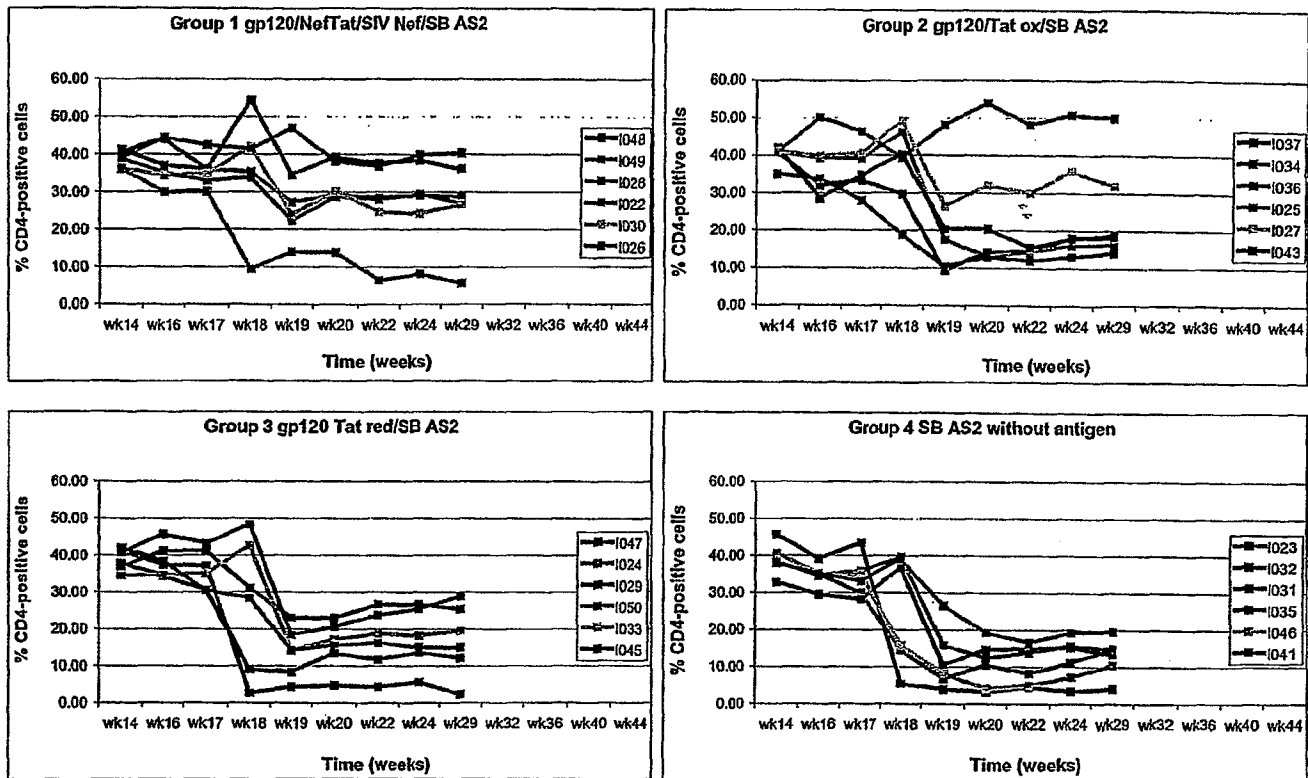
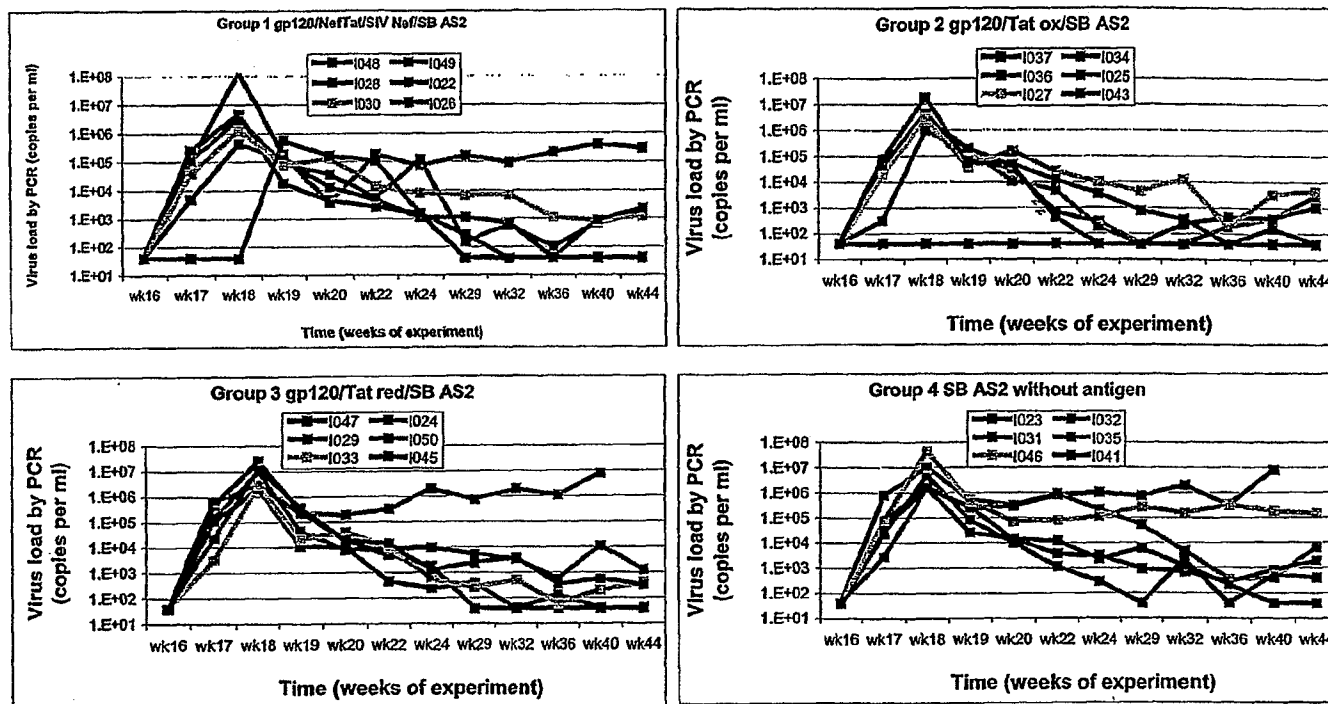


Figure 17. Monkey study 2. Analysis of SHIV plasma virus load after challenge with SHIV



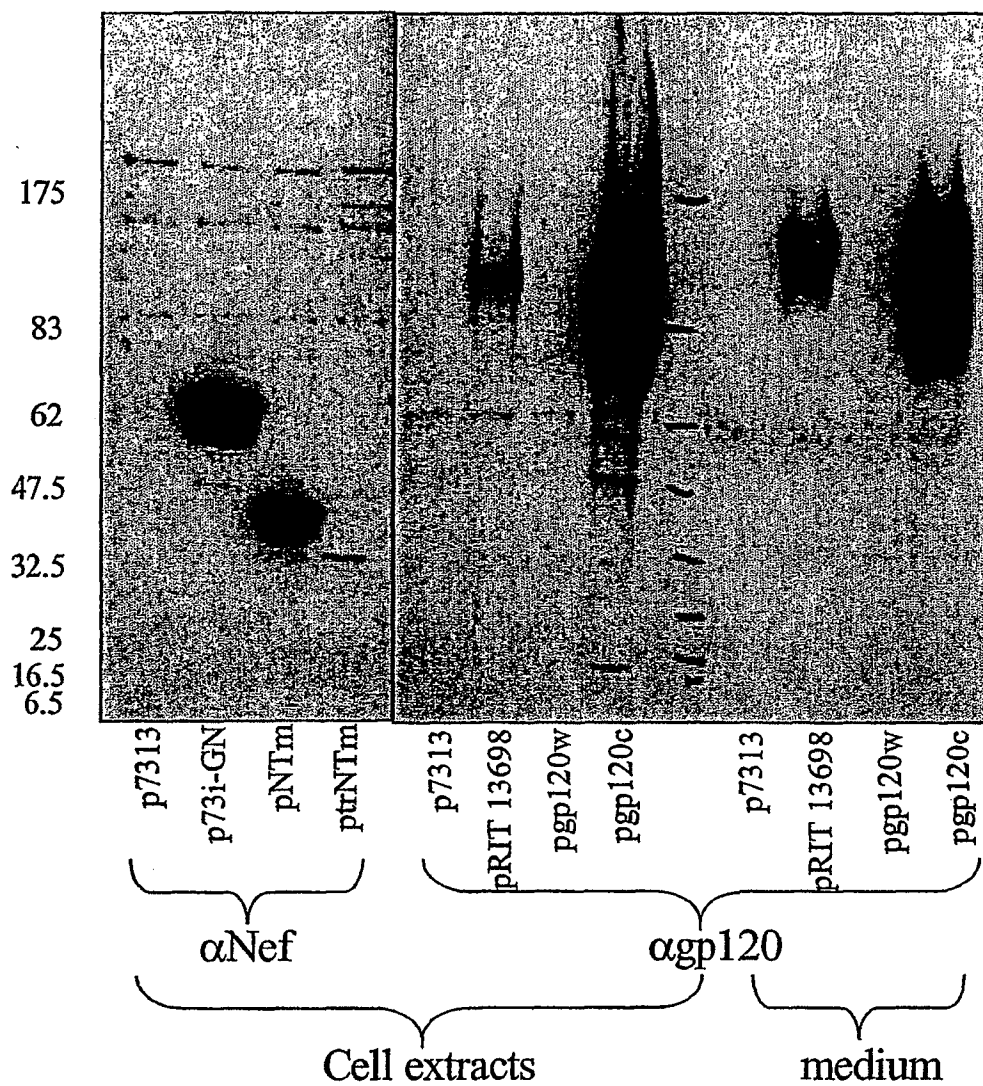


Fig. 18. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in cell extracts and supernatant medium, 24hr post transfection.



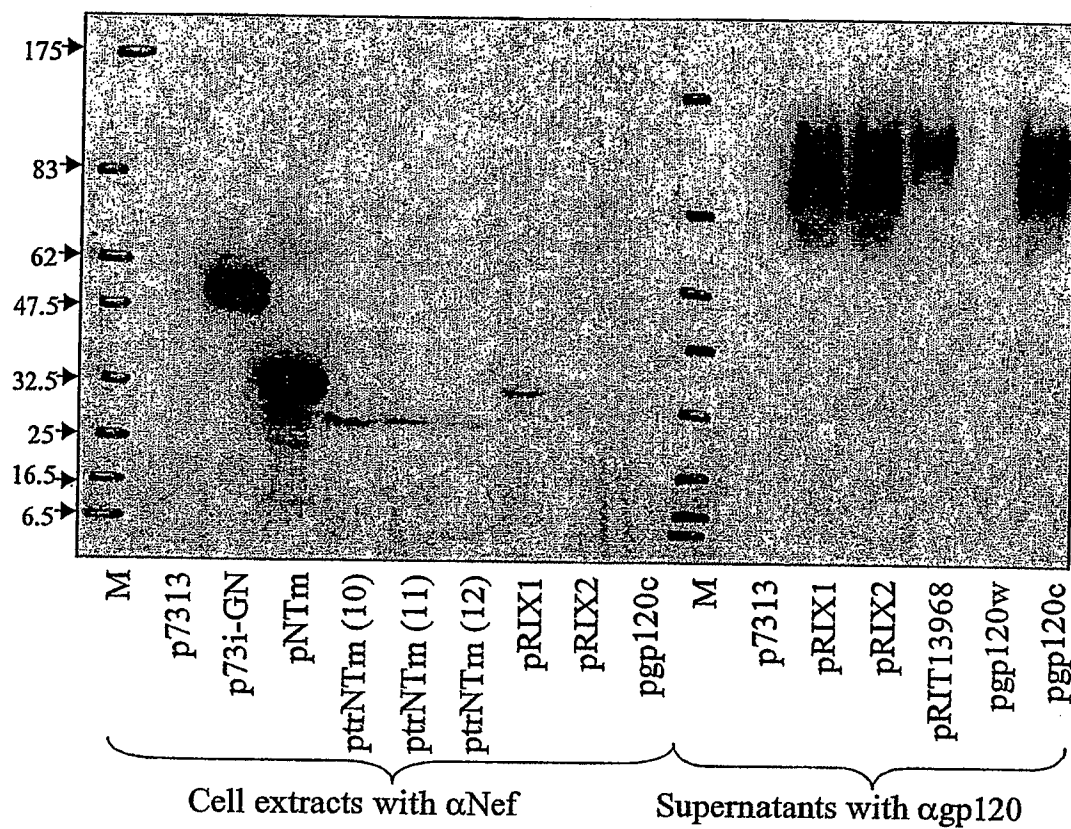


Fig. 19. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in supernatants, 24hr post transfection.

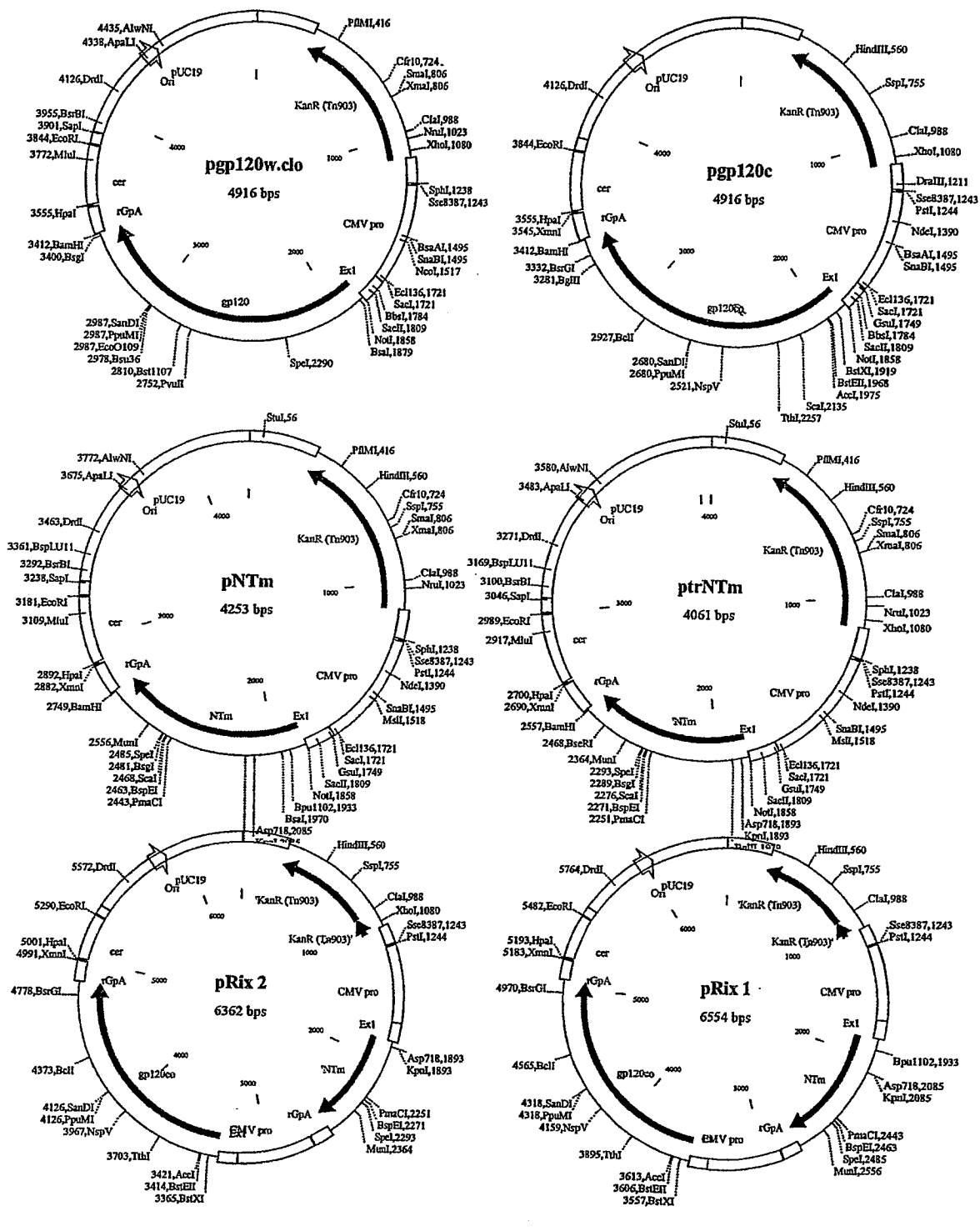


Fig.20. Plasmid Maps:

Fig. 21. Oligos for codon optimisation and sequencing of gp120:

g120-u:1 GAATTCGCGGCCGCAATGAAGGTCAAGGAGACCAGAAAGAACTACCAGCATCTGTG  
 g120-u:2 CCTGGGAATGCTGATGATCTGCTCCGCCGCCGAGCAGCTGTGGGTACCGTCTACTACGG  
 g120-u:3 GGCCACGACCACCTCTTCTGCGCGAGCGACGCCAAGGCCCTACGACACGGAAAGTGATATAA  
 g120-u:4 TTGCGTGCCCTACGGACCCCAACCCCCAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTT  
 g120-u:5 CATGGTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAGTCCCTGAAGCCCTG  
 g120-u:6 CTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACAGCAA  
 g120-u:7 TCGGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGT  
 g120-u:8 GTTTTATAATCTCGATGTGGTCCCATCGACGACGACAATGCCACCACCAAGAACAAGAC  
 g120-u:9 CATTCACTGCAACAGCAGCGTCATGACGCGAGGCCCTGCCCAAGGTGTCTCTCGAACCAAT  
 g120-u:10 CCCTGCGCGGATTCGCGATCTCAAGTGTAAACAACAGACCTTCGACGGGAAGGGCTGTG  
 g120-u:11 GCAGTGCACCCATGGCATCCGCCCGCTGAGACCCAGCTGCTGTGAACGGGTCCCT  
 g120-u:12 GATCCGGTCGGACAACCTTATGGACAACACCAAGACAATCATCGTCCAGCTGAACGAGTC  
 g120-u:13 CCGGCCATAACAACAACCCGTAAGGGCATCCACATCGGGCCTGGACGGGCCCTTCTATGC  
 g120-u:14 CGACATCCGGCAGGCCCATTCGAACCTCTCCCGCGCCAGTGGAAATAACACCTTGAAGCA  
 g120-u:15 AGAGCACTTTGGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCGAGAT  
 g120-u:16 CTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGG  
 g120-u:17 AGAGGGAAATCCACTATCACCTCCCTTGCCTGCGATCAAGCAGATCATCAACATGTGGC  
 g120-u:18 GTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCAACATCACCGGCCGTGCTGT  
 g120-u:19 CGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGCGCGCGACATGAG  
 g120-u:20 GCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCGTGGCCCCACCCGGGCCAA  
 g120-l:1 GAATTCGCGATCCTCATCTCTGCACGACGCGCGCTTGGCCCCGGGTGGGGGCCACG  
 g120-l:2 ACCACCTTGTACTTGTACAGCTCGCTCCGCCAGTTATCCCTCATGTGCGCGCCCGCGGGC  
 g120-l:3 TTCTCCGTGCGGTTGCCCTCGGTGCCCCGCTCTCTGGTGAGCAGCAGGCCGGTGATGTTG  
 g120-l:4 CCCCCGATGGGGGGGGCATAATGCGCTTCCACCTCCTGCCACATGTTGATGATCTGC  
 g120-l:5 GTGATAGTGGAGTTTCCCTCTGTGTTGTTGCCCTCGGTGCCGTTCCAGGTGGAGTTGAAG  
 g120-l:6 TAGAAGAATCGCCCCCGCAGTTGAAGGAGTGCCGACGATCTCGGGGTCTCCGCCAGAA  
 g120-l:7 GTCCTGTTTCCAAAGTGCTCTCTCAGCTTGATCACGATCTGCTTCAGGGTGTATTCCAC  
 g120-l:8 CAATGGGCCCTGCCGATGTCCGCGATGATCTTCCGGCGGCATAGAAGGCCCGTCCAGGC  
 g120-l:9 CGGGTGTTGTTGTTAGGCCCGGGTACAGTTAATGCCACAGACTCGTTTCAGCTGGACGATG  
 g120-l:10 ATGAAGTTGTCCGACCGGATCACCACTCCTCCTCAGCCAGGGACCCGTTTCAGCAGCAGC  
 g120-l:11 CGGATGCCATGGGTGCATGCACCGTGTGACGTTGGTGACAGGCCCTTCCCGTCGAAG  
 g120-l:12 AGGATCGCGAATCCGGCAGGGGCACAGTAATGGATCGGGATTGGTTGGAAGGACACCTTG  
 g120-l:13 ACGTGTCTGTTGAGTGAATGAGTCTGAAATTACGCGTCTGTTCTTGGTGGTGGCA  
 g120-l:14 ACCACATCGAGATTATAAAACAGCGCGTATTCTTCTGACCTTGTCTCTGATCGAGGTC  
 g120-l:15 TTCTTGATCTCGCCCTTCCGAATCTCTCCGGTCCAGCCGTTGCTGGTGGTGGTGGTCTG  
 g120-l:16 TCACAGTCCAGTGTACGACGAGAGGGCGTCAGCTTCACGACAGGGCTTCAGGGACTGGTCC  
 g120-l:17 TCGTGATCTGATCCACCATGTTATTCTTCCACATGTTGAAGTACTCGGTACGTTTCCC  
 g120-l:18 TTGGGGTCCGTAGGCACGCAAGCATGCGTCCGCCACAGCTTATGCACTTCCGTGTCTGTAG  
 g120-l:19 CAGAAGAGGGTGGTCTGTGGCCCTCTTCCACACAGGCACGCCGTAGTAGACGGTGACCCAC  
 g120-l:20 CAGATCATCAGCATTCACAGGAGCATGTTGCCCCAGCGCCACAGATGCTGGTAGTTCTTT

## Sequencing primers for optimised gp120

g120c-SF1 cggcgtgcctgtgtggaaggaggc  
 g120c-SF2 gacaagggtgcagaaggaataacgcg  
 g120c-SF3 cgtccagctgaacgagtcgtgtggc  
 g120c-SF4 catgtggcaggaggtgggaaaggcc  
 g120c-SR1 gtgagcagcaggccggtgatgttgg  
 g120c-SR2 cggcatagaaggcccggtccaggcc  
 g120c-SR3 catgaccgctgctgttcagtgaaatg  
 g120c-SR4 aggcacgcaagcatgctgcgcccac

## Sequencing primers for wild type gp120

g120w-SF1 ccactctattctgtgcatcagatgc  
 g120w-SF2 ccttgatgtagtacaaatagatgatg  
 g120w-SF3 ggtatacatataggaccaggagagc  
 g120w-SF4 agatgttcataaaatattacagggc  
 g120w-SR1 catatctcctcctccaggtctgaag  
 g120w-SR2 gttattccattgtgctctactaag  
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 g120w-SR4 ctacttcttgtgggttggggtctg

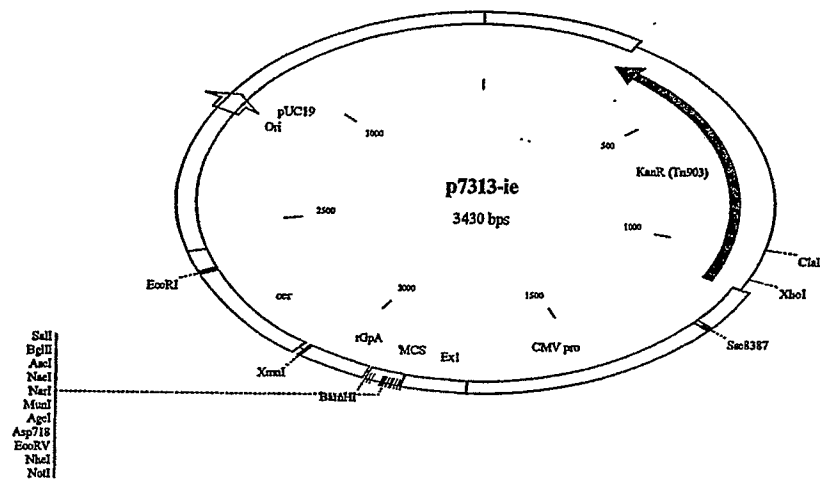


Figure 22 – Map of p7313

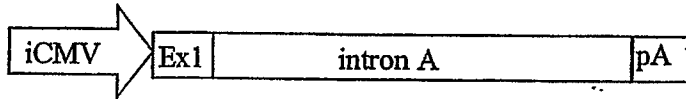


Figure 23: Typical expression plasmid for antigens

Schematic representation of the standard expression cassette used for antigen expression. The gene of interest was inserted into a plasmid 3' to a enhanced HCMV IE1 promoter (iCMV + Exon 1) (nucleotides -489 to +118 relative to the transcription start), and 5' to a rabbit beta globin polyadenylation signal (pA). The plasmid backbone additionally contained a pUC19 origin of replication and a kanomycin selection marker.

**Figure 24: Responses of immunised F1 (Balb/c x C3H) mice to gp120 peptide by IFN-gamma ELISpot at 5 days post-boost**

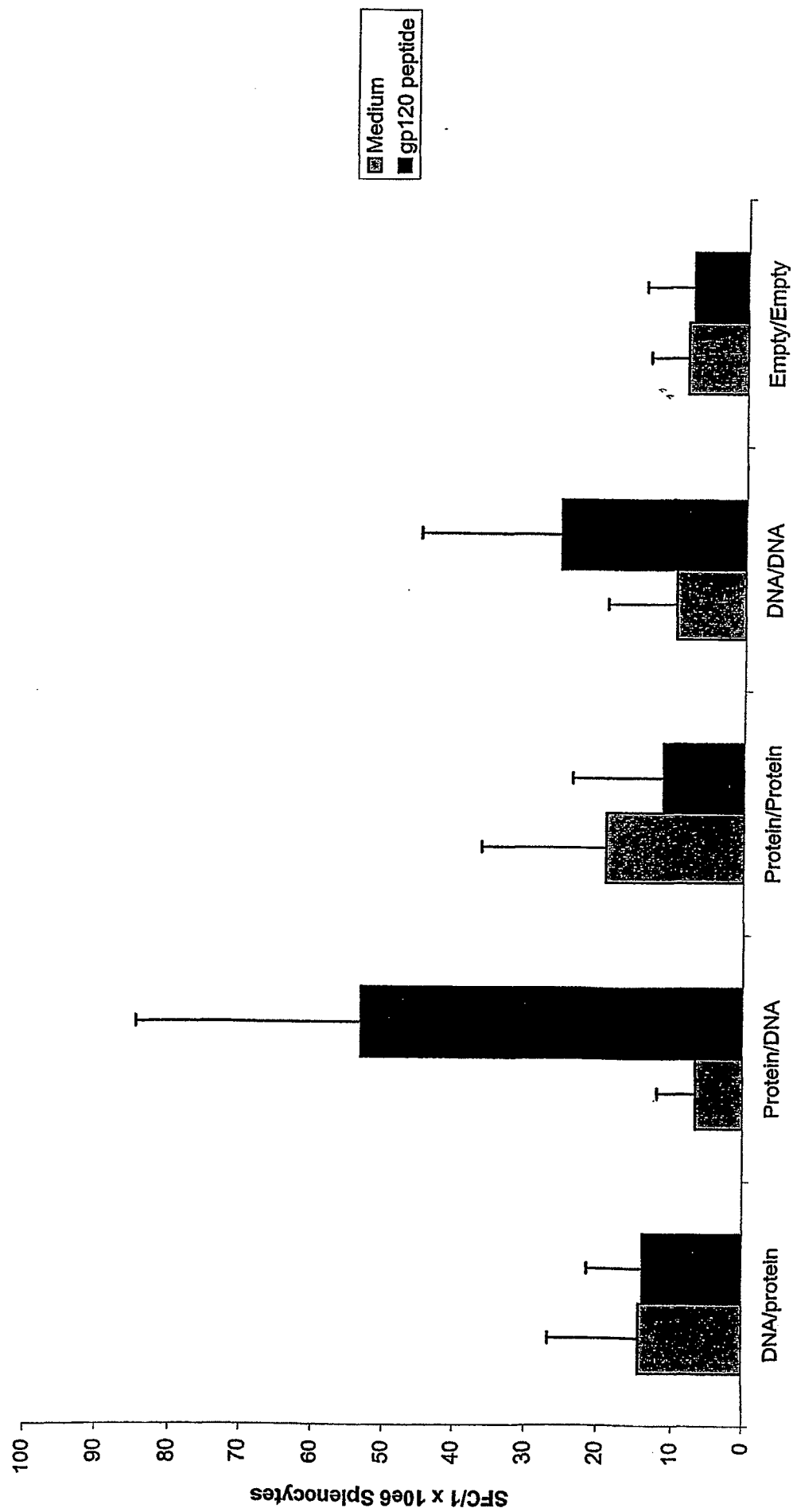
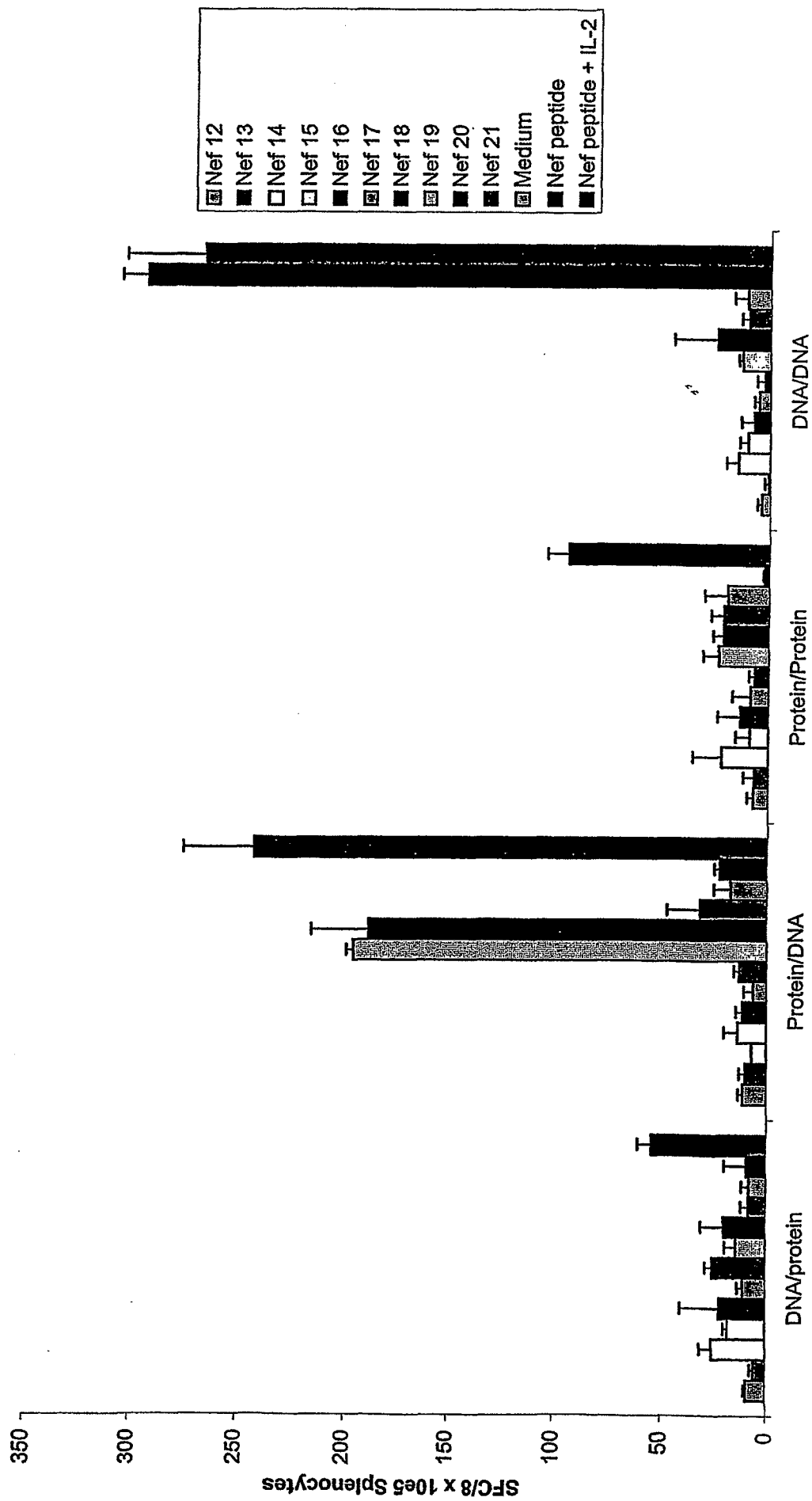
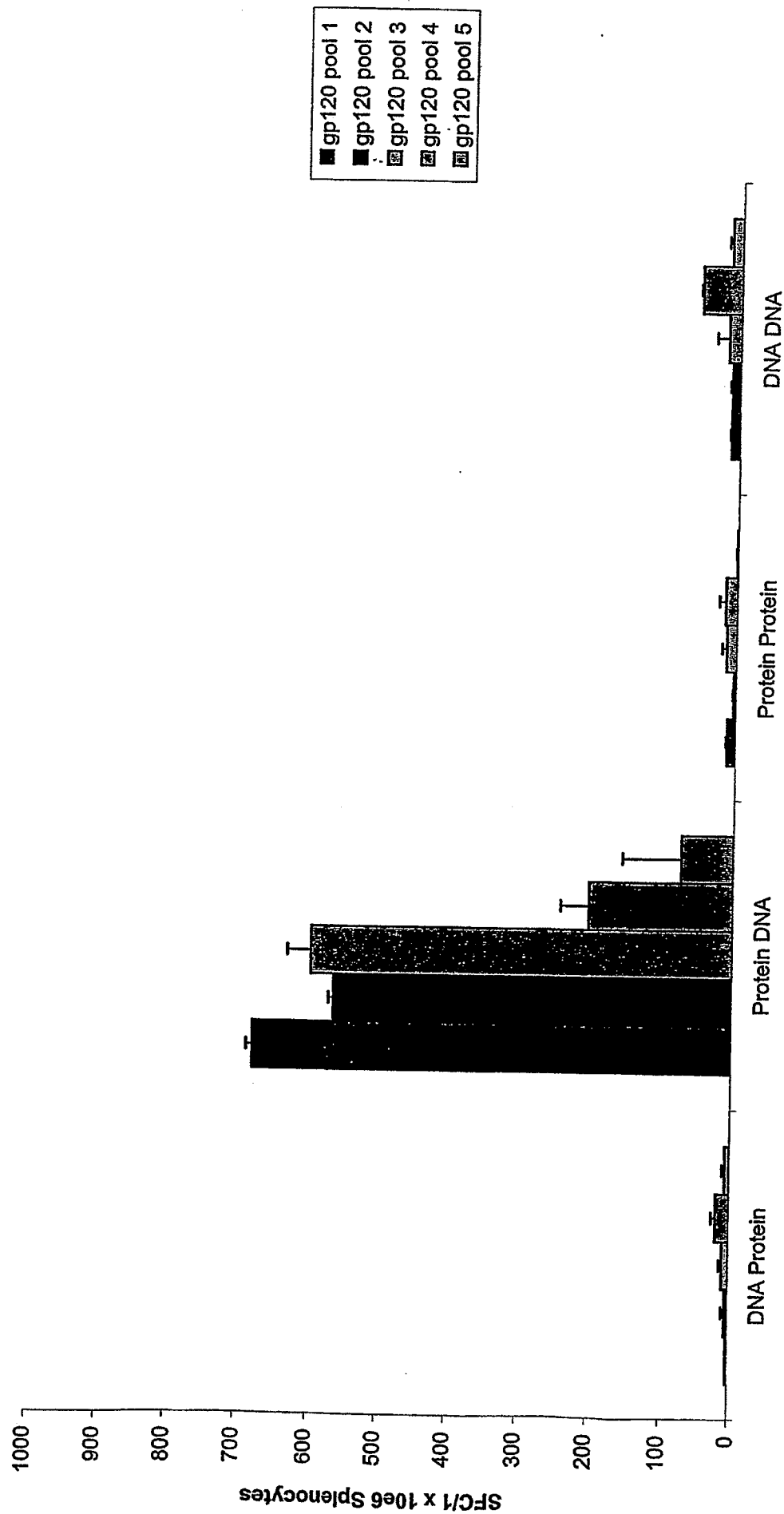


Figure 25: Responses to 20-mer Nef peptide library by IFN-gamma ELISPOT at 5 days post-boost



**Figure 26: Responses of immunised Balb/c mice to gp120 peptide pools by IFN-gamma ELIsport at 7 days post-boost**





## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/08343

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/21 A61K31/713 C12N15/49 C12N15/89 C07K14/16  
C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 16884 A (GODART STEPHANE ANDRE GEORGES ; SMITHKLINE BEECHAM BIOLOG (BE); BRU) 8 April 1999 (1999-04-08) page 8, line 12,13	1-11
X	HEYDENBURG FULLER D ET AL: "A QUALITATIVE PROGRESSION IN HIV TYPE 1 GLYCOPROTEIN 120-SPECIFIC CYTOTOXIC CELLULAR AND HUMORAL IMMUNE RESPONSES IN MICE RECEIVING ADNA-BASED GLYCOPROTEIN 120 VACCINE" AIDS RESEARCH AND HUMAN RETROVIRUSES, NEW YORK, NY, US, vol. 10, no. 11, 1 November 1994 (1994-11-01), pages 1433-1441, XP000572444 ISSN: 0889-2229	12-16
Y	the whole document	1-11, 17-22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

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## INTERNATIONAL SEARCH REPORT

International Application No

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